Nutritional aspects of breeding in birds

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Declaration

I declare that the work described in this thesis has been carried out by myself unless otherwise cited or acknowledged. It is entirely my own composition and has not, in whole or in part, been submitted for any other degree

Andrea L. Fidgett
March 2002

"It has, I believe, been often remarked that a hen is only an egg's way of making another egg."

Samuel Butler, 1877

No two alike…

For

Irene, Shona, Mum & Dad

ABSTRACT

This study set out to investigate the relationships between dietary nutrition, female quality and egg production. Representing a considerable investment of her resources, a female bird deposits all the chemical nutrients required for the growth of an avian embryo within a sealed unit over a short period of time. Variation in both the total amount of resources allocated to a clutch of eggs and the distribution of those resources within a clutch can have a profound influence on both her offspring's and her own fitness.

Cockatiels (*Nymphicus hollandicus*) were used as a model for a parrot species, to examine the interaction between the body condition of females before breeding and the quality of diet they obtain during egg formation on subsequent clutch production. Feeding a supplement of high quality nutrients had a positive influence on breeding performance. Clutch mass was 32% larger in birds that received the supplement all through the breeding cycle, versus the birds who only received a maintenance diet over the same time period. Clutch size rather than egg size was increased. High quality nutrition offered during the period of egg production did not produce significantly larger clutches. Instead there was a significant increase in clutch mass when a feeding supplement was offered during the pre-laying period, suggesting an important contribution from endogenous reserves to egg production. Feeding a supplement of high quality nutrients did not appear to affect egg mass, an attribute often used as a measure of egg quality. Chicks hatching from eggs laid by birds fed a supplement of high quality nutrients tended to gain weight and grow skeletally faster than those hatching from eggs laid by birds with no exposure to the high quality diet.

To examine interspecific variation in egg composition, eggs of 18 bird species, representing 10 avian orders, a range of dietary habits and many of the eight classes of developmental maturity of chicks at hatching, were collected and analysed. The gross composition (lipid, protein and water contents), amino acid and essential fatty acid profiles of their eggs are presented. Differences in egg composition are discussed in terms of developmental maturity of chick at hatching, and maternal diet.

Variation in egg composition and therefore quality, also occurs within a species and this was examined in detail by manipulating the egg production of lesser black-backed gulls *Larus fuscus*. Birds were experimentally induced to lay extended clutches, presumably representing their physiological and nutritional extremes of egg production, and a number of egg composition variables was measured. It is known from previous work that changes in egg composition have a substantial effect on offspring survival. The objective of this study was to examine the chemical composition of experimentally induced extended clutches in more detail, in order to elucidate which aspects of the eggs are involved in this trade-off. The gross composition (lipid, protein and water contents), amino acid and essential fatty acid profiles of their eggs are presented. An enzyme-linked immunosorbent

assay was developed to measure variation in yolk immunoglobulins, the avian equivalent of maternally-derived passive immunity, across extended laying sequences.

The weight of amino and fatty acids declined in absolute terms within an extended sequence, but relative to egg mass remained at the same concentration. Earlier laid eggs contained significantly greater quantities of vitamin E and carotenoids, a phenomenon also observed in normal three-egg clutches. Both compounds are powerful antioxidants that protect both against peroxidative damage during development and the oxidative stress associated with hatching. Caroteniods are also believed to enhance the immune system and last-laid eggs have been demonstrated to contain significantly less immunoglobulin G (IgG) than earlier laid eggs. Smaller eggs contained most major nutrients in the same proportions as larger eggs, suggesting a blueprint for egg composition exists within the female, with limited scope for variation. That the last egg laid in extended clutches was not smaller than third eggs in normal clutches indicates the probability of a minimum size threshold below which an egg is unlikely to hatch and survive. Thus differential mortality of chicks hatching from eggs laid later in a sequence may result from them having suffered more oxidative stress during development or having an increased susceptibility to pathogens.

Both the crystallographic and chemical structure of lysozyme have been documented for many bird species other than domesticated birds, yet there has been comparatively little work done on levels of lysozyme activity in wild bird eggs and their ecological importance. Investigating lysozyme activity in eggs of experimentally extended clutches laid by *L. fuscus*, within a clutch, later-laid eggs exhibited demonstrably less lysozyme activity and therefore had a lower lysozyme concentration. Since egg mass also declined significantly with laying position, these later-laid eggs were also more likely to contain less lysozyme in absolute terms. On its own, the decreasing lysozyme concentration observed in experimentally extended gull egg clutches, may not be biologically significant but combined with declines in other components of the egg already described it may contribute cumulatively to the decreased hatchability and fledging success observed in previous research.

These investigations of chemical composition of eggs, both within and between species, demonstrate the complexity of the avian female's reproductive system. Female body condition is an integral part of that system and it would appear to influence egg production long before oviposition.

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CHAPTER 1 Nutritional aspects of breeding in birds: general introduction

Reproductive characteristics and success are closely linked to food resources and many studies by nutritionists, ecologists and evolutionary biologists have sought to measure the energy and nutrients necessary for reproduction (Robbins, 1993). The obvious costs of reproduction are manifested by the nutrients contained in sperm, egg, foetus, enlarged reproductive organs (testes, ovary-oviduct, uterus, or mammary gland), or milk and the additional heat necessary for incubation in birds. While reproductive requirements are in addition to normal maintenance processes, maternal or paternal tissues can be mobilised to meet the immediate requirements. This does not lessen the requirement however, since deficits must eventually be balanced, but it offers a mechanism for distributing such high costs over a much longer period of time (Robbins, 1993). Less obvious aspects involved in the total reproductive effort are that territorial establishment, construction and defence, and courtship may have energetic costs. Furthermore, the increased time and energy spent on food acquisition and territorial defence may predispose reproducing adults to higher mortality (Nur, 1988).

Egg production

A fundamental difference between the sexes of all animals is the size of their gametes: females produce large, immobile, food-rich gametes (eggs or ova), while male gametes or sperm are tiny, mobile and consist little more than self-propelled DNA (Krebs and Davies, 1993). Birds represent ovum development and investment taken to its extreme; the avian egg is a discrete, yet complete package. Burley and Vadehra (1989), put the case more succinctly: "Avian eggs are of particular interest because, in addition to genetic information, they contain all the chemical nutrients needed for the growth of the embryo. Furthermore, this growth takes place in isolation within a hard protective shell, the only outside help being an exchange of gases, a little heat energy, and an occasional rotation. By contrast, eggs of viviparous species, such as mammals, are much smaller, do not have a hard shell, and need an external source of nutrients and possibly other chemicals for the embryo, which does not normally grow in isolation." Burley and Vadehra (1989, p1).

All of this chemical investment must be in place before the embryo starts to develop and be sufficient to cover the entire period of embryogenesis until hatching. Furthermore, in most avian species the process is repeated several times to produce not just one, but an entire clutch of eggs. Nutrient requirements for egg production are dependant on the number and size of eggs laid, their composition, and the temporal sequence of yolk and albumen synthesis. All bird species produce characteristic number of eggs in a clutch. The average size of a clutch varies from a single egg in many seabirds to eight or more in some wildfowl, and fifteen or more some game birds (Perrins and Birkhead, 1983). Size of eggs are also relatively consistent within a species, however both size and number of eggs vary with age of the female, time of laying and food availability (Cody, 1971). Average egg size in interspecific comparisons is inversely proportional to body weight - that is, smaller birds generally lay larger eggs relative to body weight than do larger birds (Carey, 1996).Variation in egg composition between species largely reflects the differing stage of embryonic development at hatching (Sotherland and Rahn, 1987). Eggs of precocial (also referred to as nidifugous) species have larger yolks than do eggs of altricial (nidiculous) birds. Because the yolk contains virtually all of the high-energy lipids, less water and more protein than albumen (Williams et al., 1982), increasing the relative size of the yolk increases the amount of energy and protein per unit of egg available for embryonic development.

The daily requirement for egg production can be estimated by proportioning the energy and protein content of the egg over the number of days required for its synthesis and deposition. Yolk synthesis within the ovary requires from 4 to 26 days, whereas albumen synthesis and deposition occurs during the 1 to 2 days that the ova passes through the oviduct (King, 1973). Estimated daily energy requirements for egg laying ranges from 29% of the basal metabolic rate in hawks and owls, which lay one egg approximately every third day, to over 200% in waterfowl, which lay a relatively large, high-energy egg every day until the clutch is complete (Robbins, 1993). Estimated daily protein requirement increases from 72% above the maintenance level in hawks and owls to 220% in waterfowl, gulls and terns. Birds meet these relatively high energy and protein requirements by reducing other non-essential costs, accumulating fat prior to laying, mobilising fat reserves as well as some protein from internal organs or muscles during laying, increasing food intake or altering the diet to include more nutritious foods, particularly insects (Jones and Ward, 1976; Hails and Turner, 1985; Perrins, 1991; Robbins, 1993).

Clutch size – how many eggs to lay?

Different mechanisms have evolved to regulate avian clutch size, with varying ecological implications. They affect the female's ability to respond to the loss of a clutch or brood (Haywood, 1993); can allow environmental factors to influence clutch size during laying (Jones and Ward, 1976), or can increase or decrease the cost of egg production for the female (Astheimer and Grau, 1985). Although these mechanisms can be manipulated experimentally they remain poorly understood aside from enabling birds to be classified as determinate or indeterminate layers. Simply put by Cole (1917), species "in which the number of eggs which will be laid in the clutch is definitely determined when laying begins" (p.504) were defined as determinate layers. Indeterminate layer were species "in which the number of eggs that will be laid depends upon stimuli received after laying has begun... As a consequence if the eggs are removed as laid the stimulus does not occur and laying continues beyond the regular clutch to an indefinite number" (Cole, 1917, p. 505).

Although many species have been categorised as one or the other, the definitions provided above are problematic, and in a re-evaluation of mechanisms for regulating clutch size, Haywood (1993) refined the categories. Determinate layers are defined as species in which extrinsic factors perceived by the female, such as eggs present in the nest, are not involved in determining either the number of large yolky follicles produced by the ovary or the number of follicles ovulated (e.g. albatrosses, auks and pigeons). In some species, the number of large yolky follicles produced is unrelated to extrinsic factors, but contact between the female and her eggs usually reduces the number of follicles ovulated and hence the clutch size. Haywood (1993) classifies these species, (e.g. penguins) as semideterminate layers. Three major types of indeterminate layers have been identified, based on the nature of the information used by the females to cease laying. Most use tactile cues developed between the female's brood patch and the eggs in the nest; this category can be further subdivided according to whether the female requires contact with her entire clutch or just a single egg is sufficient to trigger cessation of egg laying. Parasitic species, such as cuckoos, apparently control clutch size through visual information. A final cue might be via thermal means, used by megapodes that build a natural incubator rather than a conventional nest (Haywood, 1993)

The phenomenon of indeterminate laying species, whereby birds are capable of replacing eggs lost during laying or laying a second clutch if the first is lost after clutch completion,

led Lack (1947) to assume that egg production was inexpensive and many birds could easily lay larger clutches than they actually do. Lack suggested that avian clutch size has evolved to correspond to the number of young that parents can successfully provide with food at the nest (Lack, 1947). However it is now known from many studies that birds are capable of rearing more young than the number of eggs they lay (Lindén and Møller, 1989). Modifying Lack's hypothesis to encompass the concept of trade-offs over a lifetime of reproductive attempts, rather than simply based on optimising the results of one season, expands its' scope, given that parental survival and future reproductive performance may be affected by investment made in a single brood. Further refinement comes from considering the possibility that phases other than chick-rearing may also play a part in limiting clutch size. Although it has only recently received attention, the effort allocated to incubation and egg production is also important, as a growing body of research supports (see Monaghan and Nager, 1997 for review).

Thus, being the first process in the nesting cycle that requires energy and nutrient output, it is now more commonly considered that egg formation places a major physiological demand on a female bird (Martin, 1987).

Egg structure and composition

It seemed appropriate to briefly summarise the egg structure and contents within this introduction, not least to highlight the variety and complexity of materials that the female must accumulate through diet or synthesize prior to or during egg formation. Much of the description that follows (unless indicated otherwise), has been adapted from two books on the avian egg, by Romanoff and Romanoff (1949) and Burley and Vadehra (1989), which present comprehensive reviews of the literature conducted predominantly using the domestic chicken egg (*Gallus gallus domesticus*) as its model species.

Although there are large variations among species in the size and shape of their eggs, they all contain similar constituents arranged in the same way. An avian egg has three main parts: the shell plus membranes; the albumen, and the yolk (Figure 1.1).

The egg shell is an inflexible, fragile, mineralised structure that gives the egg its shape and resists deformation. A thin noncalcified layer, the shell cuticle, coats the shell. Fine pores through the shell permit entry and exit of atmospheric gases including water vapour. The

shell serves as a barrier between the embryo and the external environment, preventing invasion of potential pathogens, while serving as a structural support for the egg contents. Avian eggshells are principally composed of crystalline calcium carbonate ($CaCO_3$) and small concentrations of magnesium and phosphorous – throughout incubation the shell provides about 80% of the calcium required by the embryo for skeletal development. Just inside the shell are two membranes: a thick outer membrane (shell membrane) and a thin inner membrane (egg membrane). The two layers are separated at the wider end of the egg by the air cell, which is not present initially when the egg is laid, but appears as incubation proceeds or, for infertile eggs, as the contents contract on cooling. The two shell membranes form a boundary between the shell and egg contents. The outer shell membrane provides an attachment site for $CaCO_3$ crystals during development, while the inner membrane anchors the chorioallantoic membrane during development. Both membranes may also act as a further barrier to block microbial invasion. The membranes are primarily composed of protein and glycoprotein, with small amounts of lipid and carbohydrate.

The inner shell membrane is next to the albumen, which generally comprises the major proportion of egg content (~60% of a chicken egg). Albumen of a chicken typically contains 88% water, 11% protein and trace amounts of carbohydrate, lipid and inorganic ions (e.g. phosphorous, sodium, chloride and magnesium). Four distinct regions can be distinguished within the albumen of a fresh egg: the outer thin white next to the egg shell; the outer thick white, a gel which forms the centre of the albumen; the inner thin white, a layer near the yolk, and the chalazal layer (inner thick white), which touches the vitelline membrane and ultimately merging with the chalazae, rope-like structures holding the yolk in place. The relative proportion of albumen proteins varies between avian species, however the major proteins are usually always present and appear highly conserved within avian taxa, leading to their use in avian classification systems (Sibley and Ahlquist, 1972). The proteins have many different roles including during egg formation, the transfer of covalently bound ions, vitamins and carbohydrates from the hen's blood into the egg. Another widely described function of albumen is to provide a hostile environment for, or simply a barrier against, pathogens that have managed to permeate the primary defences of both the shell and membranes, in order to prevent them reaching the yolk. Ultimately, all albumen proteins contribute amino acids to the developing embryo when they are finally subject to catabolism.

The vitelline membrane is largely proteinaceous, but is thinner and less robust than the shell membranes and it separates the yolk from the albumen. The yolk contains most of the nutrients in the egg – a hen's egg yolk comprises approximately 50% water and 30 % lipid, with the remaining fraction largely protein. None of the yolk lipid is free - it is associated with specialised proteins. An intact egg yolk within the shell is arranged in layers (see Figure 1.1) but after being removed is fluid and heterogeneous. Most of the yolk is pigmented by carotenoids and known as yellow yolk. There is also a small colourless region of low viscosity (the latebra) connected to other colourless parts of the yolk and this material is termed white yolk (see Figure 1.1). The lipid fraction is dominated by triacylglycerides (61-72%) and phospholipids (25-36%), while cholesterol esters and miscellaneous fats make up the rest. Triacylglycerides provide about 90% of the energy used by the embryo in support of maintenance and growth of embryonic tissue, while the phospholipids become components of cell. Yolk proteins are less well understood than their albumen counterparts because of difficulties encountered in isolating them from the lipid-rich environment of the yolk. As already mentioned they play an important role in transporting nutrients (lipids, vitamins, ions and carbohydrates), both during egg formation, from the hen to the egg, and during embryogenesis, from the egg to the embryo. They also provide amino acids upon degradation. Most of the vitamins present in yolk are found in association with specific binding proteins, and several have been identified responsible for riboflavin, biotin, vitamin A (retinol), B_1 (thiamin), B_{12}, D_3 (cholecalciferol) and E (α-tocopherol). Yolk also provides the balance of minerals required for skeleton formation. In addition to all these nutrients, the yolk is also the primary source of immunoglobulins, maternally-derived antibodies that confer passive immunity to the developing embryo until it begins to actively manufacture its own.

Egg quality (... all eggs are not created equal)

Egg quality is a term widely used by avian ecologists but rarely defined in precise terms, although it is often considered synonymous with some aspect of the chemical structure or composition of the egg. As described earlier, the relative amounts of yolk and albumen vary between species and the idea that this variation may be related to developmental maturity of the hatchling was first suggested by Tarchanoff in 1884. The relative proportion of egg nutrients also varies within a species and even within the same clutch, although in many species studied to date, within-clutch variation in egg components is less

than that observed between females (Ricklefs, 1977; Arnold et al., 1991). Intraspecific egg composition has been found to vary with laying date, laying order, clutch size, location and year (Birkhead and Nettleship, 1984; Arnold et al., 1991; Williams, 1994; Nager et al., 2000). However it is still unclear whether the variation observed is subject to environmental influence or is a repeatable feature of reproduction in each species (Carey, 1996).

Nutrient limitations imposed on females before or during the laying period are often presumed to be a major source of the variation observed in egg quality (e.g. Bolton et al., 1992), but there have been few detailed studies. Much less is known or understood about the mechanisms by which these limitations would operate, and whether they affect embryonic viability, either directly or indirectly. As has already been emphasised, most of the existing data on egg composition are derived from studies of chicken eggs or other domesticated birds, particularly with regards to distribution of micronutrients, e.g. specific amino acids, essential fatty acids, vitamins or minerals (Romanoff and Romanoff, 1949; Burley and Vadehra, 1989). Although comparative data have been described for a wide range of avian species (Ricklefs, 1977; Carey et al., 1980; Sotherland and Rahn, 1987), these authors have mainly described gross egg composition e.g. proportion of yolk to albumen, or quantities of lipid, protein and water. There has been some work on amino acids (Murphy, 1994a); essential fatty acids (Speake et al., 1996, 1999); fat-soluble vitamins (Royle et al., 1999) and carotenoids (Surai et al., 2001a, 2001b). But the fact remains that few studies have described egg composition in detail, either across a broad range of wild bird species, or simply within a species under strictly defined conditions.

The success or 'quality' of an egg could also result from chemical components other than those defined as nutrients. One such 'non-nutritive' element would be the avian equivalent of maternal immunocompetence; immunoglobulins (or antibodies) deposited in the eggs. The most abundant class of antibody in avian eggs is immunoglobulin G (Rose and Orlans, 1981), often referred to as IgY (Leslie and Clem, 1969) since it is located in the yolk. Apart from a small selection of domesticated and companion bird species, there have been no studies (to the best of my knowledge) measuring directly the variation in maternal transfer of immunocompetence in any wild bird species. Another example is found in albumen; lysozyme, a protein first described by Fleming (1922). It is widely supposed to be a defence component of the egg, guarding against microbial attack of the nutrient-rich yolk.

However, although both the crystallographic and chemical structure of lysozyme has been documented for many bird species other than domesticated birds, there has been comparatively little work done on levels of lysozyme activity in wild bird eggs and their ecological importance (Jollès and Jollès, 1984).

During reproduction, the nutrient needs of laying birds include the nutrients required for maintenance of essential body components and those required for egg synthesis. Laying birds may meet their increased nutritional requirements by an increase in dietary intake, the use of body reserves, a reduction in overall metabolism of the female enabling her to redirect resources to egg formation, or any combination of the above (Murphy, 1994; Houston et al., 1995a; Houston 1997). However, the relative importance of these sources of nutrients has still not been determined for any species of wild bird (Walsberg, 1983). Energy has usually been assumed to be the limiting nutritional factor in egg production (Bolton et al., 1992), but the potential for other dietary components (vitamins, trace minerals, essential fatty acids and essential amino acids) to place restrictions on egg production should not be overlooked (Wilson, 1997). In addition, while the quality of food ingested at the time of laying is known to influence egg production (Martin, 1987; Bolton et al., 1992), the role of endogenous resources is much less understood.

The dietary experience of a female in the months preceding breeding may influence her breeding potential, and also determine her body condition at the end of breeding and hence her future fitness. Environmental factors influencing clutch size in birds have been extensively studied (Lindén and Møller, 1989). But this aspect of resource allocation between female muscle condition and egg production has received little attention and could be an important factor in the cost of egg production. If this is indeed the case, then in order to bring birds into peak breeding condition, they may require a different diet from that which is normally adequate to maintain good health - a conclusion which has important implications for avian captive breeding programmes (Houston, 1997).

Outline of Thesis

The objective of my research was to examine the relationship between nutrition and reproduction in birds in more detail, as manifested via egg production and variation in egg composition. There are published examples from gulls and finches of how diet influences female quality, which in turn influences egg quality and breeding performance. I wanted to

know whether this was also the case for a psittacine species. Many species within this taxa are threatened in the wild and as a consequence there are many captive breeding programmes in place. However, we actually know very little about the relationship between captive diets and reproduction for many wild bird species, let alone more specifically parrots.

Chapter 2 describes work using cockatiels (*Nymphicus hollandicus*) as an easily maintained, convenient model for the psittacine taxa, to examine the interaction between the body condition of female cockatiels before breeding and the quality of diet they obtain during egg formation on subsequent clutch production. Birds were fed on one of two diets prior to breeding: either a basic maintenance diet of seeds or a high quality diet of seeds plus an egg-based supplement, resulting in a higher protein content (amongst other nutrients). It was assumed that birds on the higher quality diet would be able to develop better body condition and endogenous reserves of fat and protein. Should endogenous reserves make an important contribution to egg production, such birds might be expected to show enhanced breeding performance. By feeding either the maintenance diet or the high quality diet over the laying period, I was able to address the question of whether birds which started the breeding season in poor condition can still lay large viable clutches if given high quality nutrition during the period of egg production. Or is good condition prior to breeding essential for good egg production, regardless of the quality of dietary intake during the days of egg formation?

Having examined how maternal nutrition influences egg production in a parrot species, the remainder of the thesis examines wider aspects of egg quality in terms of egg composition. For logistical reasons it was impractical to conduct these studies on cockatiels, partly because they lay such small eggs, but also due to problems encountered inducing sufficient pairs to breed, which had a direct impact on the sample sizes I could obtain. Therefore other bird species have been used. First for a comparison between species (described below), eggs of 18 bird species, representing 10 avian orders, a range of dietary habits and many of the eight classes of developmental maturity of chicks at hatching, were collected and analysed. The remainder of the work was conducted on gull eggs, specifically those of the lesser black-backed gull *Larus fuscus*. While the transition from cockatiels to gulls may not be the most obvious, working with gulls provided many advantages. Gull eggs are much larger, thereby providing more chemical material for analysis; *L. fuscus* is also an

indeterminate layer, capable of producing very large extended clutches under experimentally manipulated conditions. Furthermore, there is good evidence in this species of a trade-off between the number and the quality of eggs (Nager et al., 2000). By removing eggs as they were laid, the number of eggs produced by lesser black-backed gulls was experimentally increased beyond the normal clutch size of three, at the expense of chick viability. Neither hatching nor fledging success of the eggs was related to their fresh mass, but was related to their position in the laying sequence. This suggests that changes in egg composition had a substantial effect on offspring survival.

Chapter 3 presents an interspecific comparison of egg composition. It has been recognised for some time that the initial proportions of yolk and albumen in avian eggs vary considerably between species, most notably with respect to developmental maturity of the chick at hatching, along the altricial-precocial spectrum. Gross composition (total moisture, lipid, and protein) is known and documented for many species but, as explained already, detailed composition, comparing amino or fatty acid profiles for example, has only been determined for a very few species. In Chapter 3 I present the gross composition (lipid, protein and water contents), amino acid and essential fatty acid profiles of eggs from 18 bird species.

I then moved on to a detailed examination of intraspecific variation of egg composition in the lesser black-backed gull (*Larus fuscus*). The objective of this study was to examine the chemical composition of experimentally induced extended clutches in more detail, in order to elucidate which aspects of the eggs are involved in the trade-off between egg composition and chick survival. I wanted to investigate maternal transfer of immunocompetence in *L. fuscus*, but first I needed to compare the effectiveness of various assays for the identification and quantification of immunoglobulins in egg yolk of *L. fuscus*, and Chapter 4 describes and critically reviews the various methods employed.

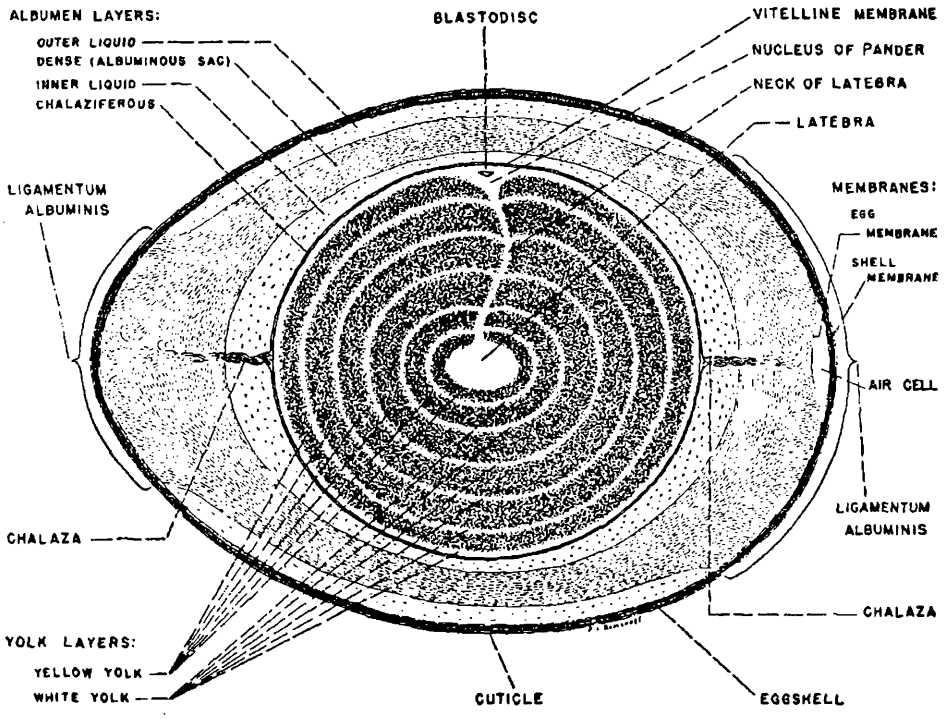
In Chapter 5, I present the results of analyses of egg composition performed for three eggs (first, third and last egg laid), from extended clutches. Experimentally extended clutches presumably represent the absolute physiological limits of egg production, and therefore nutritional limitations will be more pronounced (and detectable) with increased effort. Analyses were performed at the Central Nutritional Laboratory (Pedigree Masterfoods) and composition factors measured were water, lipid, crude protein, ash, selected essential amino

and fatty acids. Levels of total carotenoids and fat-soluble vitamins A and E were measured at the Scottish Agricultural College and are presented along with the results of my investigation into variation in maternal transfer of immunocompetence.

The final component of my examination of intraspecific variation of egg composition in the lesser black-backed gull is presented in Chapter 6, where I investigate a non-nutritive factor of egg composition; activity of the enzyme lysozyme, found in egg albumen. To the best of my knowledge, the potential for within-species variation between eggs does not appear to have been investigated for any wild bird species. I chose to examine this phenomenon in eggs collected from the lesser black-backed gull (*Larus fuscus*). I wanted to discover whether there was variation in lysozyme activity in eggs, both between eggs laid within single extended clutches, and between females. I also considered how lysozyme activity levels in this species of wild bird compared with known values for poultry, and speculate what might be the reasons for any disparity.

Much of the research contained within this thesis is largely descriptive, and in the final discussion in Chapter 7, I present an overview of my findings and describe how they fit into and further our understanding of how maternal quality and nutritional experience influences the viability of her offspring. The relevance of these findings to feeding programmes and the practice of clutch manipulations for captive bird species is discussed.

Figure 1.1 Structure of the hen's egg, shown by a section through the long axis (from Romanoff and Romanoff, 1949).



CHAPTER 2 Influence of female quality and diet on breeding performance of cockatiels (*Nymphicus hollandicus*): egg production, chick survival and growth

INTRODUCTION

Research conducted on gulls and zebra finches suggests that the body condition of the female due to her dietary experience in the weeks preceding breeding, may influence her breeding potential. It can also determine her body condition at the end of breeding and hence her future fitness (Selman and Houston, 1996; Nager et al, 2000). This aspect of resource allocation between female quality and egg production has received little attention. I wish to extend these studies to parrot species, using the cockatiel as a model for developing improved diets for breeding other psittacine species. Many parrot species are endangered in the wild and are the focus of conservation actions either through captive breeding programmes or dietary supplementation of birds in the field (Fidgett and Robert, 1993; Powlesland and Lloyd, 1994). The aim of this study was to consider the role of female condition and food quality in breeding success in a parrot species.

Egg production can place a major physiological demand on a female bird. Nutritional requirements for egg production are dependent on the number and size of eggs laid, their composition and the temporal sequence of yolk and albumen synthesis. Average egg size in interspecific comparisons is inversely proportional to body weight - that is, smaller birds generally lay larger eggs relative to body weight than do larger birds (Carey, 1996). During reproduction, the nutrient needs of laying birds include the nutrients required for maintenance of essential body components and those required for egg synthesis. Laying birds may meet their increased nutritional requirements by an increase in dietary intake, the use of body reserves, a reduction in overall metabolism of the female enabling her to redirect resources to egg formation, or any combination of the above (Murphy, 1994; Houston et al., 1995a; Houston 1997). Murphy (1994) makes a distinction between nutrient stores (where levels are elevated beyond those maintained by well-nourished non-productive birds) and reserves, which include stores *and* tissues that can be drawn upon in an emergency to reallocate nutrients when daily intake falls below requirements. The distinction is important since utilising stores may be an adaptation to avert nutritional stress, whereas relying extensively on reserves may mark the onset of nutritional stress

(Murphy, 1994). However, the relative importance of these sources of nutrients for egg production has still not been determined for any species of wild bird (Walsberg, 1983). Energy has usually been assumed to be the limiting nutritional factor in egg production (Bolton et al, 1992), but the potential for other dietary components (vitamins, trace minerals, essential fatty acids and essential amino acids) to place restrictions on egg production should not be overlooked (Wilson, 1997). In addition, while the quality of food ingested at the time of laying is known to influence egg production (Martin, 1987; Bolton et al, 1992), the role of endogenous resources is much less understood.

Birds can use pectoral muscle and other tissues as a protein source for egg production (Kendall et al., 1973; Jones and Ward, 1976; Houston et al., 1995a). A review of studies that examined female muscle weight at the time of egg formation found a significant loss in muscle weight in 21 of the 29 species examined (Houston et al., 1995c), suggesting that the use of tissue protein to assist with egg production may be widespread. Recent studies on zebra finches (*Taeniopygia gutatta*) found that food intake did not increase during egg production but the decline in female body reserves of protein and fat over this period was sufficient to account for most of the nutrients in a clutch of four eggs (Houston et al., 1995a). The use of labelled amino acid demonstrated that there is an enhanced loss of amino acids from muscle tissue of laying zebra finches, and that these are used in the formation of egg proteins (Houston et al., 1995b). Further studies found that muscle condition of female zebra finches before the start of the breeding season has a profound effect on the number, size and hatchability of eggs (Selman and Houston, 1996). This phenomenon was attributed to muscle proteins forming a substantial contribution to egg formation (Houston et al., 1995a; Houston et al., 1995b).

The dietary experience of a female in the months preceding breeding may influence her breeding potential, and also determine her body condition at the end of breeding and hence her future fitness. Environmental factors influencing clutch size in birds have been extensively studied. But this aspect of resource allocation between female muscle condition and egg production has received little attention and could be an important factor in the cost of egg production. If this is indeed the case, then in order to bring birds into peak breeding condition, they may require a different diet from that which is normally adequate to maintain good health - a conclusion which has important implications for avian captive breeding programmes (Houston, 1997).

There is a common assumption that birds, given sufficient choice, possess 'nutritional wisdom' to select an appropriate dietary balance. Murphy (1993) has shown that in fact birds have a limited ability to discriminate between diets which differ in important nutrients but whether this is sufficient to maximise egg production is not known. In captive settings, naturalistic or 'cafeteria style' feeding, whereby animals are fed a range of foods types rather than a fixed ration of manufactured pellets relies heavily on the concept of 'nutritional wisdom', unless quantities are of each item are strictly monitored (Allen, 1982). Further, birds in the wild have the opportunity to select a far greater diversity of food items than usually on offer in a captive situation. Wild birds are known to make significant changes to their diet in order to satisfy specific requirements; calcium-rich items being sought out during the period of egg formation is one such example. Captive birds do not have this option and are dependent on being provided with diets that are deemed nutritionally adequate based on the birds' likely requirements. Information about the nutritional needs of birds is generally limited to domesticated species selected for egg and meat production (see Table 2.1, Requirements determined by National Research Council, 1994). However, production goals for these (predominantly poultry) species are somewhat different to that of endangered species captive breeding programmes.

As a group, parrots contain a significant number of threatened species (Bennett and Owens, 1997), some of which are the focus of captive breeding programmes. Parrot species have been the subject of nutritional studies, partly as a result of their popularity as pets, the budgerigar *Melopsittacus undulatus* being an example (Earle and Clarke, 1991), while other, more threatened species have been studied in captivity in order to improve their husbandry. Diet was implicated as a factor in the morbidity of St. Lucia parrots (*Amazona versicolor*) maintained at the Jersey Wildlife Preservation Trust as part of a captive breeding programme for the species, prompting an investigation into their nutrition (Fidgett and Robert, 1993). However, even psittacine species are generally grouped together despite ranging from budgerigars to macaws, the spectrum of size encompassing a variety of eating habits which are mainly vegetarian but vary in the proportions of fruits, nuts, seeds and other plant fractions consumed. Roudybush and Grau (1986), feeding diets of varying protein concentrations to cockatiel chicks, found that a diet containing 20% protein (on a dry weight basis) was the minimum required for maximal growth and this figure has been used to develop diets for other captive psittacines (Baer and Ullrey, 1986). Of the essential

amino acids, only recommended levels for lysine (0.8% on a dry matter basis) have been determined, again for cockatiels (Roudybush and Grau, 1991). Earle and Clarke (1991) found that budgerigars had quite high energy requirements to maintain basal metabolism, but that the apparent metabolizability of the nutrients in their seed-based diet was adequate to cope with these demands. Mixtures typically comprising millet, sunflower, canary seed, wheat, hemp, safflower and others seeds are commonly available as commercial diets for parrots species. Yet in many instances they are likely to be nutritionally inadequate since most seeds are deficient in certain essential amino acids, calcium and other trace minerals and tend to be rich in fat (Ullrey et al, 1991). The nutritional composition of a selected range of seeds is provided in Table 2.2.

Study Animals

Cockatiels have been the subject of debate as to their taxonomic position within the Order Psittaciformes (Holyoak, 1971; Smith, 1975; Adams et al., 1984; Saunders et al., 1984). Despite being much smaller and more coloured than other cockatoos, they share more common morphological characters used to distinguish the cockatoos from parrots – a crest, lack of 'green' plumage and young that possess natal down. Thus they have commonly been placed in a subfamily of the Cacatuidae (Adams et al., 1984; del Hoyo et al., 1997), a conclusion supported by biochemical investigations (Adams et al., 1984). No extensive field studies of the species have been undertaken, and what is known of their ecology has largely been published as general observations. Approximately 30cm in length and weighing between 80-100 g, they are sexually dimorphic, cavity nesting, monogamous birds endemic to Australia. Both parents participate in incubation and chick care and clutch sizes are typically three to seven eggs with a breeding season usually between August and December. Several clutches may be laid in a single season. They are widely distributed throughout the interior of the continent, inhabiting grassland, savannah woodland and cultivated land (Forshaw, 1989; Vriends, 1989; del Hoyo et al., 1997). Although principally a ground-feeding granivore, feeding on 29 seed types, in the only study of feeding ecology conducted in a grain-growing area, four of these were found to be the grain-crops sunflower, sorghum, wheat and oats (Jones, 1987). Of these, sorghum comprised 60% of total crop contents (Jones, 1987). As such they are regarded as a pest species by many farmers and some are destroyed under permit (del Hoyo et al., 1997).

Cockatiels are easy to breed in captivity, particularly when provided with the appropriate environmental conditions (Millam et al., 1988). Although force-pairing birds will lead to some breeding activity, mate familiarity enhances reproductive activity (Yamamoto et al., 1989) as does early rearing experience; parent-reared birds performing better than hand-reared birds (Myers et al., 1988).

Aims of this chapter

The aim of this chapter is to examine the interaction between the body condition of female cockatiels before breeding and the quality of diet they obtain during egg formation on subsequent clutch production. Birds were fed on one of two diets prior to breeding: either a basic maintenance diet of seeds or a high quality diet of seeds plus an egg-based supplement, resulting in a higher protein content (amongst other nutrients). It was assumed that birds on the higher quality diet would be able to develop better body condition and endogenous reserves of fat and protein. Should endogenous reserves make an important contribution to egg production, such birds might be expected to show enhanced breeding performance. It is already well known for many birds that dietary quality during the period of egg production can influence egg and clutch size, and so birds were also placed on either the maintenance diet or the high quality diet over the laying period, producing the four treatment groups shown in Figure 2.1. This allowed me to address the question of whether birds which started the breeding season in poor condition can still lay large viable clutches if given high quality nutrition during the period of egg production. Or is good condition prior to breeding essential for good egg production, regardless of the quality of dietary intake during the days of egg formation?

Breeding trials were conducted over two years. In the first season I collected eggs laid by the females under different dietary regimes and studied their composition. In the second year I allowed some of the eggs to be incubated so as to study hatchability and growth rates of the young. But the basic experimental design was similar between seasons. My original intention was to obtain measures of body condition of the females at different stages of the breeding season from a series of body measurements, body moulding and body mass. However, the process of capture and handling the birds was found to be highly stressful to all concerned, was likely to jeopardise breeding, and was therefore abandoned. Experience with other species has shown that in small birds it is not possible to detect physical changes in condition caused by different dietary regimes, even though these induce major

differences in breeding performance (Selman and Houston, 1996). Therefore the omission of this aspect of the project is unlikely to have been important.

MATERIALS & METHODS

Housing and Husbandry

Pairs of cockatiels were housed in galvanised steel wire cages (0.6 x 1.8x 0.6m) that were mounted on the walls and arranged in columns three cages high (Figure 2.2). Each cage contained two or three wooden perches. Food, water, oyster grit and cuttlefish bone were refreshed daily and available continuously in bowls placed in the centre of the cage (Figure 2.3). The birds were fed a maintenance diet of sunflower seeds and 'Haith's aviary mixture' (John E. Haith, Cleethorpes, UK). The aviary mixture contained yellow millet, canary seed, black rape seed, linseed and Japanese millet (Table 2.2 details the proportions of seed within the mix), and it was fed in a separate bowl from the sunflower seed. The birds were usually also fed one of following items daily; seasonal leafy green vegetables (e.g. curly kale, broccoli, spinach), carrot or sweetcorn cobs. At least once a week the birds were also given sprays of millet, or branches cut from apple or willow trees, as a means of enrichment.

Season 1, Egg production & egg quality

Pairs of cockatiels that had been maintained on the diet of sunflower seeds, Haiths' aviary mix and the additional items described above were randomly divided into two groups. One group of 14 pairs continued on the maintenance diet. The other group (also of 14 pairs) were fed the maintenance diet supplemented with an egg mash; homogenised whole, hardboiled chicken eggs without the shell, mixed in equal parts on a fresh weight basis with white rusk. The birds were kept as pairs in the cages, allowing both males and females free access to all of the food items presented. These diet regimes were in place for 10 weeks during which time the birds were not observed attempting to copulate or lay eggs. After 10 weeks on the experimental diet environmental conditions were then altered to stimulate breeding conditions by providing nest boxes and increasing photostimulation from 9 hours (08:00 - 17:00) to 15 hours (06:00 - 21:00) (Millam et al., 1988; Yamamoto et al., 1989). In the wild, cockatiels are found throughout Australia and it has been noted that northern populations have a variable breeding season that is largely dependant upon rainfall (Forshaw and Cooper, 1981), rather than changes in daylength or temperature commonly

used by temperate avian species (Immelmann, 1971). However the budgerigar (*Melopsittacus undulatus*) and zebra finch (*Taeniopygia guttata*), both tropical-to subtropical species like the cockatiel, respond to photoperiodic stimulation in captivity (Shellswell et al., 1975; Bentley et al., 2000). So although nestbox provision and female condition are likely to be more important in initiating breeding in captivity, response to changes in photoperiod cannot be discounted.

At this point pairs in each of the two groups were again randomly allocated to one of two further treatments: they continued to be fed their existing diet regime or were transferred to the opposite one. So half the birds who had not received supplement were now fed it, and half of those who had been fed the egg supplement no longer received it. The experimental design, resulting in four groups each containing 7 pairs, is summarised in Figure 4. This second, 'laying' phase of the experiment continued for a further five weeks, until the peak of egg production had passed; 95% and 100% of all eggs had been laid within this five week period in Season 1 and 2 respectively (Figure 2.4).

Nest boxes were checked daily first thing in the morning and any eggs laid were removed and replaced with a dummy egg to prevent the female from laying extended clutches. Once removed, each egg was identified and marked by pen, weighed to the nearest 0.001g and maximal length and width measured. The eggs were then stored in a cold room (~4°C) until prepared for egg composition analysis, a process which occurred within 48 hours of collection. This was done by cracking the egg open around the airspace, breaking off small fragments of shell around the airspace to make a hole, which had to be large enough to pour the egg contents onto a glass petri dish without splitting the yolk (Figure 2.5). The yolk was separated from the albumen with the aid of a spatula, being extremely careful to pull the albumen away without rupturing the delicate yolk membrane. The yolk was weighed to 0.001g. The entire egg shell, complete with the removed fragments and was weighed 'wet' and then allowed to air-dry to a constant weight. The dry shell weight was also measured to 0.001g and albumen weight was then calculated by subtracting yolk and dry shell weights from the total fresh weight of the egg.

Season 2, Egg production & chick quality

The same feeding regimes were in place, as described previously and illustrated in Figure 1 and, as in Season 1, there were 28 breeding pairs, resulting once more of seven pairs in

each of 4 treatment groups. Birds were allocated to treatment groups with the opposite feeding regime to what they had experienced during Season 1. Therefore birds in Group 1 during Season 1 were now in Group 4, and so on, with the exception of four new female birds. These were brought in for Season 2 to replace three birds that died and one female who laid eggs all year round and was clearly not suitable for the breeding trial. One of the new breeding pairs was allocated to each treatment. Otherwise, the breeding pairs remained unchanged from the previous year.

To mark the start of the laying period, nestboxes were attached to the cages and the day length was extended from 10 hours (0700-1700), to 14 hours (0600-2000). All the birds were immediately curious about their nest boxes and the nest boxes were checked daily for eggs. All eggs were removed in order that they could be weighed to the nearest 0.001g and maximal length and width measured. The first egg in the laying sequence was removed for analysis and replaced by a dummy. The second and third eggs were also weighed & measured, but then marked (with pencil or non-toxic marker) and replaced in the nest to be incubated as normal. All subsequent eggs were removed and replaced with dummies, keeping only the last egg in the sequence for analysis (see Figure 6 for a summary). Eggs for analysis were stored in a cold room (~4°C) until prepared to measure yolk and shell weight, a process which occurred within 48 hours of collection. Eggs that were not used for fostering or compositional analysis were discarded.

Cockatiel incubation normally lasts 18-21 days, usually starting when the third egg has been laid. Cockatiel pairs were allowed to incubate their own two eggs in the nest for 15-16 days from the day that the third egg was laid. However, in order to control for parental effects on growth of young, the eggs were allocated to another breeding pair for rearing on a random basis before they hatched. Taking into account variation in incubation length, eggs were carefully removed from the parental nest box and placed in the foster nest box at day 15-16 to ensure the process was completed before the chick was due to hatch. Each pair of birds incubated two eggs but hatched eggs from two different clutches (date matched so that hatching was synchronous). Thus each pair of eggs laid were initially incubated together with a sibling, but then hatched and reared in separate nests, although they were always reared with an egg laid by a bird from the same treatment.

To monitor growth and development, several measurements were taken on the day of hatching and at three day intervals for a five week period until day 35, since chicks normally fledge at this age. Weight was measured to 0.0001g; head-bill length, tarsus length, wing limb length (length of radius and ulna), and wing and feather growth (as before, plus length of longest primary feather) were measured to 0.1mm using callipers (Figure 2.7).

So, as an example: if Pair 1 from Treatment 1 lay 6 eggs: 1a-f then eggs a, d, e and f were removed, replaced with dummies, weighed and measured but only a and f were further separated to determine yolk size etc. Eggs b and c were incubated for 15-16 days, and then fostered separately to new parents (randomly allocated from Treatments 1-4), but always raised with another egg laid by a female from the same treatment group (Treatment 1 in this example).

In this way, eggs from any one treatment group were reared by parents from all the treatment groups. By rearing eggs in pairs with non-siblings, but similar treatments, I removed the competitive advantage one treatment might be predicted to have over another if chicks were mixed (sibling rivalry was not the focus of this research). Pairs of eggs allocated to foster parents were also matched according to predicted hatch date, to reduce any competitive advantage in the nest from hatching first.

RESULTS

I will briefly describe the results from each season and refer to tables where those details have been summarised. There was however, a degree of overlap between seasons and it was possible to pool data from both years to examine the effect the various treatments on females breeding for the first-time, and those that bred in successive years.

Season 1, Egg production

Of the 28 breeding pairs assigned to the four treatment groups, 14 pairs laid eggs. Of those, two females used the cage floor to lay their eggs and a third used her food bowl, resisting all attempts made to induce them to use the nest boxes provided. Since it was not possible to control for normal clutch size in these conditions, the data from these females were excluded from further analysis. The remaining 11 females were distributed across all four treatment groups: Group 1, n=2; Group2, n=3; Group 3, n=2; Group 4, n=4.

Table 2.3a summarises the key egg production parameters for each treatment group. The sample sizes spread over the four treatments were insufficient for statistical comparison looking at differences between each of the groups, however, certain trends were apparent. There was a tendency for total clutch mass to be larger for birds that had been fed supplement versus those who had not. Within the supplement-fed birds, those fed during the pre-laying period (versus during laying), tended to produce larger clutches, and the group which had been fed supplement throughout the entire trial period produced the largest total clutch mass (Group 4, ξ clutch mass = 35.8g $\pm$ 2.22). The overall average egg mass was 5.23g (s.d. $\pm$ 0.41g, n = 60). The mean egg mass per clutch for each group did not differ greatly from this figure, nor did the mass of first laid eggs in each treatment group (Table 2.3a). The number of eggs laid per clutch varied between treatments to produce the increase in clutch mass described above, rising from 4.5 eggs ($\pm$ 0.5) for Group 1 (birds that were never fed supplement) to 6.5 ($\pm$ 0.65) in Group 4 (birds that had been fed supplement throughout the trial). Figures 2.8a-c demonstrate these trends for Season 1 graphically. The number of days before the first egg was laid was highly variable, with Group 4 birds tending to lay much later than the treatment groups. There tended to be more gap days in larger clutches.

Season 1, Egg quality

The parameters used to evaluate quality from eggs collected in Season 1 are summarised in Table 2.3b. There do not appear to be any differences between treatment groups in either absolute mass or proportion of shell. It appears there is a tendency for actual fresh mass of yolk to decline in eggs from birds that were supplemented and this trend is also reflected when the yolk mass is expressed as a proportion of total mass. Correspondingly, since egg mass does not differ between groups, if yolk mass declines, these eggs have slightly more albumen, both on an absolute and relative basis.

Season 2, Egg production

As before 28 breeding pairs were assigned to the four treatment groups and 19 pairs laid eggs, an increase on the previous season. However, five females either used the cage floor to lay their eggs or made no attempt to incubate, a behaviour not observed in the previous season and the data from these females were excluded from further analysis. The

remaining 14 females were distributed across all four treatment groups: Group 1, n=4; Group2, n=2; Group 3, n=4; Group 4, n=4.

Table 4a summarises the key egg production parameters for each treatment group. The sample sizes spread over the four treatments were again insufficient for statistical comparison but trends apparent in Season 1 were not so marked. Total clutch mass tended to be larger for birds that had been fed supplement versus those who had not, but was also highly variable (Group 4, ξ clutch mass = 28.99g ± 9.31). The overall average egg mass was 5.26g (s.d. ± 0.57g, n = 70). The mean egg mass per clutch for each group did not differ considerably from this figure, nor did the mass of first laid eggs in each treatment group (Table 2.4b). The number of eggs laid per clutch was more constant than in Season 1, but once more it was egg number rather than egg mass that contributed to the larger clutch mass laid by birds from Group 4. Figures 2.8a-c demonstrate the trends observed in Season 2 for clutch mass, egg mass and clutch size. The number of days before the first egg was laid was less variable between groups than in Season 1, as was the number of gap days within the clutch.

Season 2, Egg quality

Since I was investigating chick quality in Season 2, there are only composition data from first and last eggs in clutches and these are summarised in Table 2.4b. There do not appear to be any differences between treatment groups in either absolute mass or proportion of shell, yolk or albumen.

Egg Production, First-time Breeding Birds Seasons 1 & 2

The egg production of all females laying for the first time over both seasons is presented in Figures 2.8a-c. Being fed supplement during the prelaying period produced significantly heavier clutches (two-way ANOVA, $F_{1,14} = 6.37$; $P = 0.024$), than being supplemented while laying ($F_{1,13} = 2.22$; $P = 0.161$), and there was no significant interaction between the two periods of the breeding cycle ($F_{1,12} = 0.01$; $P = 0.924$). Heavier clutches were due to more eggs ($F_{1,14} = 5.60$; $P = 0.033$), , rather than larger ones ($F_{1,14} = 0.79$; $P = 0.388$). There was no significant difference between the treatment groups in the number of days until the first egg was laid ($F_{1,14} = 0.72$; $P = 0.410$). Neither was there a significant correlation between the number of days birds had been fed supplement before laying their

first egg and the mass of that first egg (Figure 2.9). However the number of gap days (that is days when no egg was laid in between the first and last egg of a clutch), was very significantly shorter for birds in the prelaying supplement treatment groups ($F_{1,14}$ = 10.00; P = 0.007). Number of gap days was also strongly correlated with clutch size (Figure 2.10).

Egg Production, Repeat Breeding Birds Seasons 1 & 2

Figures 2.11a-c present summary data for nine pairs that bred in successive seasons. As described earlier in the methods, in Season 2 pairs were placed on the opposite treatment to that which they received in Season 1. Based on the evidence already described for first-time breeding birds (that diet consumed during prelaying exerts a stronger influence on egg production), birds have been grouped according to the treatment they were on during the prelaying period in Season 1. Unfortunately the samples sizes preclude statistical comparison. As might be expected from previous analyses, mean egg mass per clutch shows no evidence of variation between seasons. Clutch mass and clutch size of birds fed supplement is larger in Season 2 than the previous year (Season 1), when they were not supplemented. There is also a tendency for birds not supplemented in Season 2 to lay smaller, lighter clutches.

Egg Quality, First-time Breeding Birds Seasons 1 & 2

To determine whether there was a sequence effect in the size of eggs produced as the clutch progressed, for each clutch the slope of the regression of egg number plotted against egg mass was used to test if the mean slopes of all the clutches was significantly different from 0. Egg mass did not change with egg position in the laying sequence (T-test, T = -0.68, P = 0.51, n = 11). So it was possible to combine data from Season 2, where only first and last eggs from clutches had been sampled, with data from Season 1 where all eggs were sampled. These data are summarised in Table 2.7. In terms of absolute quantities, there was no difference between the treatment groups in dry weight of shell, or wet weight of yolk or albumen. Egg components could also be considered as proportions relative to whole egg mass. These values were arcsine transformed before being analysed by two-way ANOVA (Table 7). On this basis there appeared to be a significant effect of interaction between the treatments on the proportion of yolk present in eggs ($F_{1,12}$ = 5.26; P = 0.041), while birds fed supplement during the prelaying period tended to have slightly less albumen ($F_{1,14}$ = 4.13; P = 0.061).

Season 2, Chick quality

Almost half (45%) of the b- and c- eggs laid and incubated as part of the experimental design in Season 2 did not hatch (Table 2.8). Whether an egg hatched or not did not appear to be related to differences in egg mass ($T = 0.38$, $P=0.71$, $Df = 19$); laying date ($T = -1.80$, $P=0.08$, $Df = 24$); or the number of days the pair had received supplement prior to commencing laying ($T = 0.09$, $P=0.93$, $Df = 23$). On inspection of eggs that did not hatch when anticipated, only one egg contained a fully-formed embryo. The presence of blood vessels in other eggs suggested they had been fertile, but that any embryos had died early in development.

Incubation lasted from 17-24 days (ξ 18.6 $\pm$ 1.8, n= 15) and all the chicks that hatched survived to fledge around 35 days later. No significant relationship was observed between egg mass and initial hatch weight (Figure 2.12). Given the small number of chicks hatched (n = 15), I have not been able to analyses the data statistically and have chosen to consider them as individuals within each treatment group, despite the issue of pseudo-replication given that most of the chicks hatched were one of a pair of siblings that shared the same birth parents. However, as described in the methods section, although incubated together by their birth parents, just before hatching each egg was transferred to a new nest for hatching and rearing and siblings were raised independently with different foster parents. Preliminary review of the growth patterns for the various parameters measured (chick mass, head-bill length, tarsus length, wing-limb length and primary feather growth) revealed that chicks hatched from eggs laid by birds from treatment Group 1 appeared to lag in comparison with all the other treatment groups. Therefore the growth trajectories in Figures 2.13-2.17 are based on whether the birth parents had access to the egg supplement at any point during the reproductive cycle, versus those that did not.

Although there was no difference in initial hatching mass, it appeared that early growth tended to be suppressed in chicks from eggs laid by non-supplemented parents, while chicks from supplemented birds perhaps grew heavier, faster (Figure 2.13). All chicks fledged at approximately the same time and ultimately reached the same weight by Day 35. Measures of chick skeletal size (head-bill, tarsus and wing limb length) also suggest that chicks from supplemented birds tended to grow skeletally larger, faster, although once more, chicks from eggs laid by non-supplemented parents had caught up by Day 35.

However, feather growth in chicks from supplemented birds always appeared to be slightly greater, and although similar at Day 35, the values did not converge.

DISCUSSION

Although statistical tests have been performed, there were obviously some limitations given the small sample sizes involved, which impacts on the interpretation of the data. It is possible that responses to the different treatment groups may have been masked or remained undetected because so few birds actually bred. However some trends were apparent, and I will discuss these first before returning to why so few birds bred.

Egg Production

Feeding a supplement of high quality nutrients had a positive influence on breeding performance - clutch mass was 32% larger in birds that received the supplement all through the breeding cycle, versus the birds who only received a maintenance diet over the same time period. Birds tended to lay more, rather than larger eggs and the observation that clutch size rather than egg size increases when supplementary food is provided has been observed in a number of other species, e.g. American coots (Arnold et al., 1991), lesser black-backed gulls (Hiom et al., 1991) and Nazca boobies (Clifford and Anderson, 2001).

I was interested in examining the interaction between the body condition of female cockatiels before breeding and the quality of diet they obtained during egg formation on subsequent clutch production. Selman and Houston (1996) showed that zebra finches fed a high-protein diet for two weeks before pairing, showed a smaller loss of muscle protein during laying than birds on a low-quality (non-supplemented) diet. Although unable to measure body condition directly, because of the potential negative effects that the stress of handling might have on their breeding, the experimental design meant it was still possible to assess the effect of body condition indirectly. Birds given high quality nutrition during the period of egg production did not lay significantly larger clutches (in terms of clutch mass) than those who received no supplement at all. However, there was a significant increase in clutch mass when a feeding supplement was offered during the pre-laying period. Since the extra nutrients from the high quality diet were not available while eggs were being formed (first eggs were laid ~ 7 days after the last day that pre-laying

supplement was offered), this suggests an important contribution from endogenous reserves to egg production (Murphy, 1996). Body condition is likely to have been a major contributory factor since its has long been recognised that muscle contains the largest reserve of mobilizable protein in the bodies of animals (Ashley and Fisher, 1967; Fisher 1967). Williams and Martyniuk (2000), demonstrated changes in organ masses (heart and liver) during egg production in zebra finches which were more closely associated with dietary manipulations than muscle. Birds that received supplement throughout the entire reproductive period performed significantly better than all of the other groups and this did not appear to be simply an additive effect of being supplemented for the longest period of time. The capacity for digestive and metabolic adaptations have been mostly demonstrated in species of Galliformes and Anseriformes, although recent investigations of digestion and metabolism in wild birds suggest more widespread genotypic and phenotypic plasticity in nutrient use (see Murphy, 1996; Klasing, 1998 for reviews). However, the physiological mechanisms and control thereof are far from understood. Treatment of non-breeding females with exogenous estradiol (to mimic elevated estrogen levels and trigger onset of egg production), caused no change in organ mass (Williams and Martyniuk, 2000). Nevertheless, it is still plausible that the sustained high plane of nutrition in the Group 4 birds triggered as yet identified endocrinal and/or enzymatic pathways responsible for partitioning resources for egg production. We can therefore conclude that good condition prior to breeding is essential for good egg production, regardless of the quality of dietary intake during the days of actual egg formation.

Egg quality

Feeding a supplement of high quality nutrients did not appear to affect egg mass, an attribute often used as a measure of egg quality. Large eggs tend to produce larger chicks that in turn are more likely to survive to fledgling age (Williams, 1994). However, an increase in egg mass is not a consistently observed phenomenon in studies involving supplementary feeding (Houston, 1997). In my study the maintenance diet may not have been sufficiently 'poor' nutritionally when compared against the supplement. In other words, it was not the objective of this experiment to determine the lowest nutritional threshold for egg production, but rather whether egg production could be enhanced. So birds on the maintenance diet were not subject to a nutritional challenge, given that seed was available *ad libitum*. Egg size is a distinctive characteristic of the individual female and each bird lays eggs that vary in weight within fairly narrow limits (Romanoff and

Romanoff, 1949), egg size and therefore mass probably constrained by the width of the oviduct. Although there are a high number of cockatiels kept in captivity, they are mainly domestic pets, and as such there are very little data published on egg size for this species and it may be that size is highly conserved with little variability between individuals.

Egg composition was compared between groups in terms of absolute quantities and relative proportions. However, since the egg mass did not vary significantly between groups, both absolute and relative measures show the same trends. Shell weight was consistent across the groups. There was a trend for eggs from birds that had not been supplemented to have a slightly higher percentage of yolk and conversely (since egg mass did not vary between treatments), birds that were supplemented laid eggs that contained slightly more albumen. Although the yolks from eggs laid by Group 1 birds tended to be bigger, they may not have been better, since I only weighed the fresh yolk and did not correct for water content. Water content of yolk is known to vary widely between species (Ricklefs, 1977; Carey et al., 1980; Sotherland and Rahn, 1987). It also varies within species, both between females and within clutches (Birkhead and Nettleship, 1984; Nager et al., 2000). Therefore yolks may have been slightly larger but their contents potentially more dilute. Yolks were measured fresh and frozen whole without drying, with the intention of analysing the nutrient composition in more detail at a later date – this unfortunately proved impractical for logistical reasons.

Yolk mass has been described as a more specific indication of egg quality (Williams, 1994), given that it could benefit both structural growth of the chick as well as its stored nutrient reserves. But in practice, variation in yolk content mainly involves changes in lipid content rather than in protein content (Alisauskas, 1986; Arnold, 1989), a finding consistent with the fact that large eggs give rise to chicks with greater nutrient reserves at hatching rather than being structurally larger (Williams, 1994). Weight of shell, yolk and albumen of cockatiel eggs were the only components investigated for this study to provide a measure of 'quality'. The potential for more specific nutrient limitations (e.g. essential amino or fatty acids, fat-soluble vitamins, caroteniods), to be manifested in eggs, is explored later in Chapter 5 of my thesis.

Chick survival & growth

Chicks hatching from eggs laid by birds fed a supplement of high quality nutrients tended to gain weight and grow skeletally faster than those hatching from eggs laid by birds with no exposure to the high quality diet. The advantage in growth was short-lived given that by the time of fledging (at Day 35), so called 'non-supplemented' chicks had caught up with their 'supplemented' counterparts. This is most likely due to the fact that all the breeding birds rearing chicks had the egg supplement provided *ad libitum* for five weeks post-hatch. While having weight and morphometric measurement taken the chicks were regularly observed with their crops full of the high quality egg supplement, regurgitated for them by their foster-parents since they did not leave the nest box until around Day 35. Growth spurts to counteract nutritional deficiencies earlier in developmental history are well recognised, but such compensatory growth strategies are also associated with a variety of costs that are often not evident until much later in adult life (see Metcalfe and Monaghan, 2001 for review). Following the development of the cockatiels past fledgling age was beyond the scope of this study. One final trend that emerged was the tendency for 'supplemented' chicks to grow longer primary feathers, and was one trend where the 'non-supplemented' chicks did not catch up by Day 35, although the growth rates were not significantly different. This picture is interesting since using whole egg as a supplement means it has the potential to provide in excess, the appropriate sulphur-containing amino acids, methionine and cystine, necessary for keratin production (Klasing, 1998).

A significant proportion of the eggs laid did not develop. The pattern was not random, since it related to specific females, however it may not have been caused by a physiological failing of the female. The eggs may not have been fertilised, or incubation behaviour by either or both the sexes may have been deficient. Only one of the eggs developed fully then failed to hatch, and all the remaining chicks that did hatch survived to fledge. The fact that so few birds bred was disappointing and no obvious reason for this failing presents itself. Since mate familiarity enhances reproductive activity (Yamamoto et al., 1989), as far as was possible, I let birds form natural pairs; certainly they were exposed to their potential partners for a minimum of 12 weeks prior to the experiments commencing. However I had no knowledge or control of their early rearing experience which has also been demonstrated to affect reproductive activity; parent-reared birds performing better than hand-reared birds (Myers et al., 1988).

Implications for captive breeding of psittacines

From the results presented, the quality of diet a cockatiel receives before laying can have a profound influence on breeding success, manifested not just as larger clutch sizes but also, I would tentatively suggest based on a limited data set, via improved growth and development of the subsequent chicks that hatch. The mechanisms by which improved nutrient provision are managed by female birds with respect to reproduction, are as yet unclear, although it is reasonable to suggest that muscle (or some other tissue mass) may act as a repository for the limiting nutrients. Precisely which nutrients may be limiting egg production is the subject of further study within this thesis, although not for cockatiels. It is an area of research that requires further investigation to best serve the conservation purposes of captive breeding programmes, both for psittacines and indeed other endangered avian taxa.

Table 2.1 Selected nutrients and the range of dietary concentrations required by growing and egg-laying pheasants, quail, ducks, geese and turkeys (Scott, 1973; NRC, 1994)

	Physiological status		
Nutrient	**Early (rapid) growth phase**	**Egg Production**	**Maintenance (maximum)**
Energy, kJ ME/g	11.5-12.5	11.3-12.1	-
Protein (%)	16.0-30.0	14.0-24.0	8.0-14.0
Amino acids (%)			
Arginine	1.1-1.9	1.1	0.47
Histidine	0.4-0.6	0.4	0.12
Lysine	0.6-1.5	0.8-1.0	0.56
Isoleucine	0.6-1.1	0.8	0.31
Leucine	1.3-2.0	1.3	0.53
Valine	1.0-1.4	0.8	0.4
Methionine + Cysteine	0.5-1.1	0.7	0.38
Phenylalanine + Tyrosine	1.6-1.8	1.3	0.49
Threonine	0.9-1.1	0.7	0.32
Tryptophan	0.2-0.4	0.2	0.06
Inorganic elements (%)			
Calcium	0.4-1.7	1.9-3.8	0.44
Phosphorus	0.3-1.0	0.6-1.1	0.21
Sodium	0.1-0.25	0.1	0.12
Potassium	0.4-0.8	0.6	0.35
Chlorine	0.15	0.15	0.12
Trace elements(mg/kg)			
Magnesium	150-500	350	150-350
Manganese	<12-95	60-70	?
Zinc	25-210	40-60	25
Iron	130	130	120
Copper	13	13	5
Selenium	0.1-2.8	0.1-0.2	0.1
Fat-soluble vitamins (IU/kg)			
Vitamin A	825-13000	3300-13000	2200
Vitamin D_3	700-2000	1500	480
Vitamin E	9-50	25	9
Water-soluble vitamins (mg/kg)			
Riboflavin (B_2)	2.7-8.0	3.5	2.0
Nicotinic acid	21-72	60	15
Pantothenic acid	8-40	15-16	8
Choline	<1200-2000	1100-2500	1045
Vitamin B_{12}	0.001-0.005	0.005	0.001
Thiamine (B_1)	1.6-2	?	1.6
Pyridoxine (B_6)	2.0-6.0	?	2.0
Biotin	0.2-0.3	?	1.25
Vitamin C	?	?	?
Fatty acids (%)			
Linoleic acid	1.0-1.5	1.0-1.5	<<1.0

Table 2.2 Composition of dehusked seeds fed to cockatiels separately or as part of Haith's Aviary Mix (Taylor, 1997 and unpublished data provided by the Central Nutritional Laboratory, Waltham Centre for Pet Nutrition).

Common name	yellow millet	canary seed	black rape seed	Japanese millet	striped sunflower
% Haith's aviary mix[1]	**45**	**30**	**15**	**5**	
Nutrient composition					
Moisture (g/100g)	10.9	9.75	5.5	10.9	7.70
CP (g/100g)	14.8	17.03	22.8	12.6	13.2
Fat (g/100g)	5.3	9.6	17.5	4.3	30.0
Ash (g/100g)	2.0	5.05	4.1	2.7	2.9
Nitrogen free extract (g/100g)	69.0	58.57	50.1	69.5	46.2
Inorganic elements (mg/kg)					
Calcium	149	390	2837	145	140
Phosphorus	3548	4240	7282	2605	360
Magnesium	1358	1406	2693	1140	200
Manganese	11	56	19	10	1.8
Iron	204	58	85	45	33
Potassium	1857	2609	7364	2108	660
Sodium	219	69	54	62	5
Copper	19.5	6.74	4	6	1.3
Zinc	32	30	41.5	22	2.7
Fat-soluble vitamins					
Vitamin A (IU)	<0.1		0.1	<0.1	<20
Vitamin E (mg/100g)	<0.1		85		24.8
Water-soluble vitamins (mg/100g)					
Riboflavin (B_2)	1.0	n/a	2.8	0.31	0.43
Nicotinic acid	50	9.8		73	4.25
Pantothenic acid	26	5.7		3.9	1.23
Vitamin B_{12}	.0002	0.0037		0.0038	0.00
Thiamine (B_1)	2.7	n/a	4.6	4.35	0.38
Pyridoxine (B_6)	2.6	0.96		2.6	0.48
Fatty acids (g/100g)					
Linoleic acid	4.5	2.38	2.9		16.0

[1] Mixture also contains linseed at 5%, but composition data was unavailable

Table 2.4a Reproductive output of cockatiels during Season 2 (1999), summarising various measures of egg production. Unless stated otherwise, values are means ± standard error, with (n) based on the number of females who actually bred on each treatment.

	Treatment			
	1	2	3	4
Feeding regime pre-laying/laying[1]	No Sup/ No Sup	No Sup/ Sup	Sup/ No Sup	Sup/ Sup
Number of females	7	7	7	7
Number that bred[2] (n)	4	2	4	4
Total eggs laid	20	9	20	21
Clutch mass (g)	25.73 ± 4.05	21.86 ± 0.15	26.43 ± 1.02	28.99 ± 9.31
Clutch size (egg no.)	5.00 ± 0.71	4.50 ± 0.50	5.00 ± 0.41	5.25 ± 1.31
Egg mass/clutch (g)	5.11 ± 0.13	4.91 ± 0.51	5.35 ± 0.30	5.28 ± 0.32
Mass of first egg	5.24 ± 0.20	4.85 ± 0.25	5.10 ± 0.30	5.27 ± 0.31
No. of days to first egg	7.8 ± 1.3	11.5 ± 3.5	13.75 ± 3.8	10 ± 1.5
No. of gap days	5.5 ± 1.6	3 ± 0	3.3 ± 0.3	4.5 ± 0.7

Table 2.4b Cockatiel egg composition, used as indicators of egg quality, measured during Season 2 (1999).

	Treatment			
	1	2	3	4
Feeding regime pre-laying/laying[1]	No Sup/ No Sup	No Sup/ Sup	Sup/ No Sup	Sup/ Sup
Number that bred[2] (n)	4	2	4	4
Total no. of eggs laid	20	9	20	21
Shell dry wt. (g)	0.34 ± 0.01	0.31 ± 0.03	0.35 ± 0.01	0.35 ± 0.02
as % of fresh egg wt.	6.8 ± 0.3	6.4 ± 0.2	6.6 ± 0.1	6.8 ± 0.2
Yolk wet wt. (g)	1.16 ± 0.03	1.28 ± 0.12	1.32 ± 0.09	1.12 ± 0.11
as % of fresh egg wt.	22.9 ± 0.6	26.4 ± 0.1	24.6 ± 0.2	21.4 ± 1.6
Albumen wet wt. (g)	3.35 ± 0.12	3.08 ± 0.28	3.49 ± 0.3	3.56 ± 0.24
as % of fresh egg wt.	66.1 ± 0.8	63.6 ± 0.1	64.9 ± 1.1	68.3 ± 1.1

[1]Feeding regime pre-laying/laying, where 'no sup' means no supplement fed and 'sup' indicates the supplement was fed.

[2]Excluding females that did not lay in their nest box, as described in the text.

Table 2.5 Treatment groupings used in 2-way ANOVA to determine effect of timing on feeding supplement during reproductive cycle.

	No Supplement	Supplement
Pre-laying (PL)	Groups 1 & 2	Groups 3 & 4
Laying (L)	Groups 1 & 3	Groups 2 & 4

Table 2.6 Reproductive output of all birds breeding for the first-time, combining data from Seasons 1 and 2 (1998 and 1999). Unless stated otherwise, values are means ± standard error, with (n) based on the number of females who actually bred on each treatment.

	Treatment						
	1	2	3	4	Prelay[3]	Lay[3]	Interaction[3]
Feeding regime pre-laying/laying[1]	No Sup/ No Sup	No Sup/ Sup	Sup/ No Sup	Sup/ Sup			
Number of females	14	14	14	14			
Number that bred[2] (n)	3	4	3	6			
Total eggs laid	12	19	15	36			
Clutch mass (g)	20.96 ± 3.22	23.75 ± 1.54	26.68 ± 1.54	31.43 ± 3.17	$F_{1,14}$ = 6.37; P = 0.024*	$F_{1,13}$ = 2.22; P = 0.161	$F_{1,12}$ = 0.01; P = 0.924
Clutch size (egg no.)	3.67 ± 0.33	4.75 ± 0.25	5.00 ± 0.58	6.00 ± 0.63	$F_{1,14}$ = 5.60; P = 0.033*	$F_{1,13}$ = 3.91; P = 0.070	$F_{1,12}$ = 0.11; P = 0.745
Egg mass/clutch (g)	5.22 ± 0.17	5.01 ± 0.25	5.42 ± 0.37	5.26 ± 0.19	$F_{1,14}$ = 0.79; P = 0.388	$F_{1,13}$ = 0.60; P = 0.451	$F_{1,12}$ = 0.02; P = 0.899
Mass of first egg	5.17 ± 0.13	5.08 ± 0.26	5.05 ± 0.19	5.48 ± 0.20	$F_{1,14}$ = 1.28; P = 0.276	$F_{1,13}$ = 0.05; P = 0.835	$F_{1,12}$ = 0.03; P = 0.859
No. of days to first egg	10.7 ± 2.2	15.3 ± 1.3	15.0 ± 5.0	16.4 ± 3.2	$F_{1,14}$ = 0.72; P = 0.410	$F_{1,13}$ = 0.68; P = 0.423	$F_{1,12}$ = 0.33; P = 0.576
No. of gap days	5.0 ± 2.5	2.8 ± 0.3	3.7 ± 0.7	4.6 ± 0.7	$F_{1,14}$ = 10.03; P = 0.007**	$F_{1,13}$ = 2.29; P = 0.154	$F_{1,12}$ = 0.12; P = 0.730

[1]Feeding regime pre-laying/laying, where 'no sup' means no supplement fed and 'sup' indicates the supplement was fed.

[2]Excluding females that did not lay in their nest box, as described in the text. [3]Results of two-way ANOVA

Table 2.7 Measures of cockatiel egg composition combining data from Seasons 1 and 2 (1998 and 1999), used as indicators of egg quality. Unless stated otherwise, values are means ± standard error, with (n) based on the number of females who actually bred on each treatment.

	Treatment						
	1	2	3	4	Prelay[3]	Lay[3]	Interaction[3]
Feeding regime pre-laying/laying[1]	No Sup/ No Sup	No Sup/ Sup	Sup/ No Sup	Sup/ Sup			
Number that bred[2] (n)	3	4	3	6			
Total no. of eggs laid	12	19	15	36			
Shell dry wt. (g)	0.36 ± 0.01	0.34 ± 0.03	0.34 ± 0.01	0.34 ± 0.01	$F_{1,14}$ = 1.96; P = 0.183	$F_{1,13}$ = 0.74; P = 0.404	$F_{1,12}$ = 0.18; P = 0.676
as % of fresh egg wt.	6.8 ± 0.2	6.7 ± 0.2	6.48 ± 0.1	6.6 ± 0.2	$F_{1,14}$ = 3.49; P = 0.083	$F_{1,13}$ = 0.18; P = 0.675	$F_{1,12}$ = 1.59; P = 0.231
Yolk wet wt. (g)	1.24 ± 0.08	1.27 ± 0.06	1.38 ± 0.13	1.22 ± 0.04	$F_{1,14}$ = 0.10; P = 0.752	$F_{1,13}$ = 1.09; P = 0.314	$F_{1,12}$ = 1.91; P = 0.192
as % of fresh egg wt.	23.8 ± 1.0	25.4 ± 0.4	25.4 ± 1.4	22.5 ± 1.0	$F_{1,14}$ = 1.03; P = 0.328	$F_{1,13}$ = 0.37; P = 0.551	$F_{1,12}$ = 5.26; P = 0.041*
Albumen wet wt. (g)	3.40 ± 0.10	3.22 ± 0.17	3.54 ± 0.3	3.56 ± 0.19	$F_{1,14}$ = 1.71; P = 0.212	$F_{1,13}$ = 0.26; P = 0.616	$F_{1,12}$ = 0.15; P = 0.707
as % of fresh egg wt.	65.2 ± 0.5	64.3 ± 0.44	64.9 ± 1.7	67.5 ± 1.1	$F_{1,14}$ = 4.13; P = 0.061	$F_{1,13}$ = 0.60; P = 0.451	$F_{1,12}$ = 3.58; P = 0.083

[1]Feeding regime pre-laying/laying, where 'no sup' means no supplement fed and 'sup' indicates the supplement was fed.

[2]Excluding females that did not lay in their nest box, as described in the text.

[3]Results of two-way ANOVA; percentage values having been arcsine transformed before analyses performed.

Table 2.8 Summary of variables measured relating to hatching success and chick development. Unless stated otherwise, values are means ± standard error, with (n) based on the number of females who actually bred on each treatment.

	Treatment			
	1	2	3	4
Feeding Regime[1]	No Sup/ No Sup	No Sup/ Sup	Sup/ No Sup	Sup/ Sup
Number of females	7	7	7	7
Number that bred[2] **(n)**	4	2	4	4
Eggs incubated	7	4	8	8
Eggs hatched	4	2	6	3
Chicks fledged	4	2	6	3
Incubation period (days)	18.5 ± 0.3	21 ± 3	17.7 ± 0.2	19 ± 1.0
Hatch				
Mass (g)	4.29 ± 0.25	4.71 ± 0.92	4.87 ± 0.45	5.33 ± 0.81
Tarsus length (mm)	7.75 ± 0.48	7.50 ± 0.50	8.50 ± 0.34	8.67 ± 0.67
Winglimb length (mm)	9.13 ± 0.43	8.50 ± 0.50	9.42 ± 0.45	9.83 ± 0.44

[1]Feeding regime pre-laying/laying, where 'no sup' means no supplement fed and 'sup' indicates the supplement was fed.

[2]Excluding females that did not lay in their nest box or attempt to incubate their eggs, as described in the text

Figure 2.1 Design of experiment to investigate the effect of diet supplementation on egg production in cockatiels.

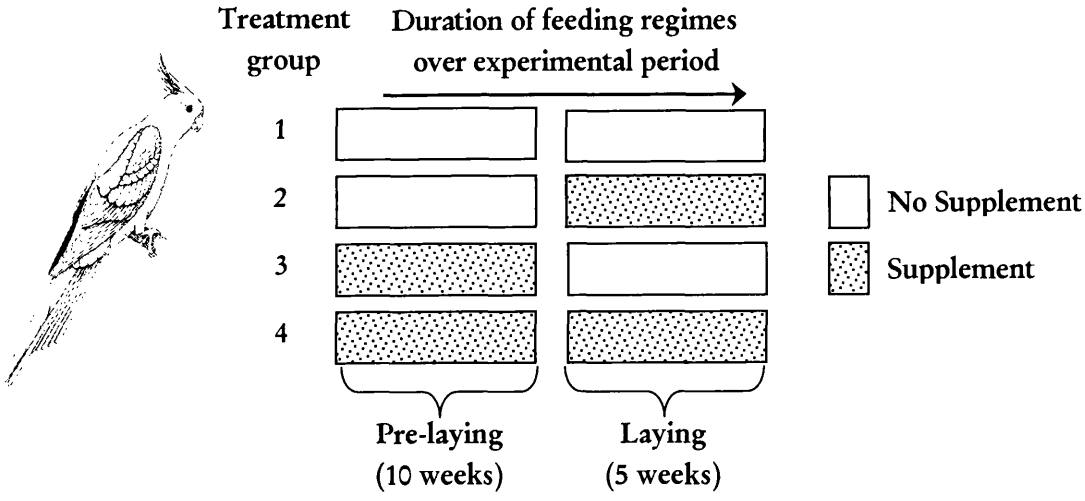


Figure 2.2 Photograph of the cockatiel housing, showing the columnar arrangement of their cages, which were mounted onto the walls. This picture was taken during the laying period and also shows the nest boxes attached to the front of the cages.

Figure 2.3 Food, water and mineral supplements provided in every cage daily.

Figure 2.4 Pattern of egg production by cockatiels after nest boxes were provided.

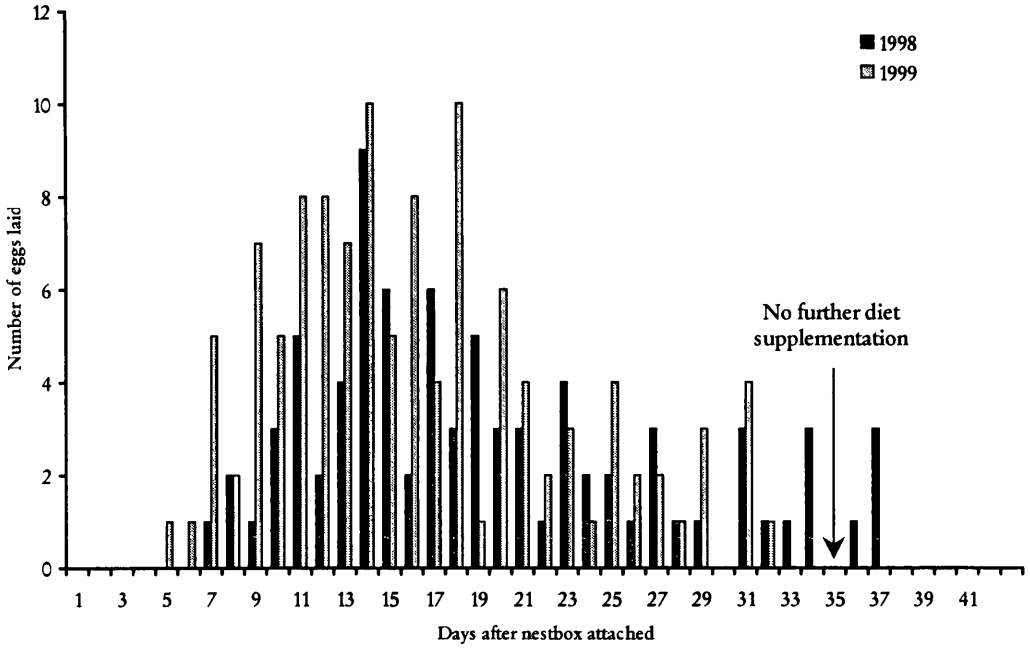


Figure 2.5 Photograph showing a cockatiel egg being processed to measure egg composition in terms of shell, yolk, and albumen weight.

Figure 2.6 Design of experiment to investigate effect of diet supplementation on chick survival and growth.

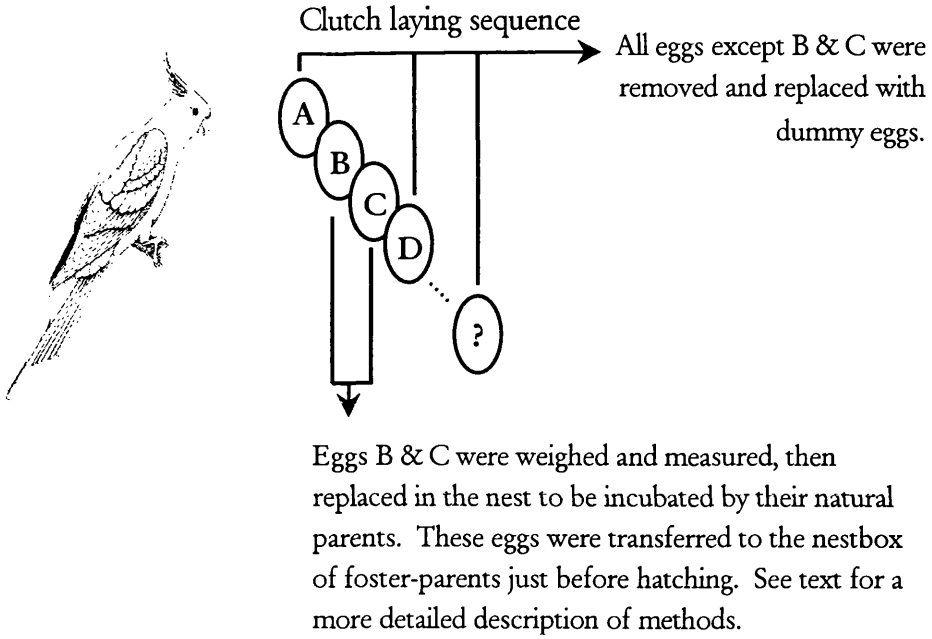


Figure 2.7 Photographs showing nest box inspection and measurements of chick growth.

a. Birds were generally easy to displace from the nest box during the early stages of incubation, but close to hatching and while chicks were in the nest, the task of checking nest box contents was more difficult.

b. (Below left) The balance was tared with the plastic pot and padding in place, after which it was simple to weight chicks even when they were considerably older than the one pictured, ~7 days old. **c.** (Below right) Measurements were taken at regular intervals from hatching of wing-limb growth (1) wing-limb length. Once feathers started to emerge, primary feather growth was measured from tip of wing limb to longest feather (2).

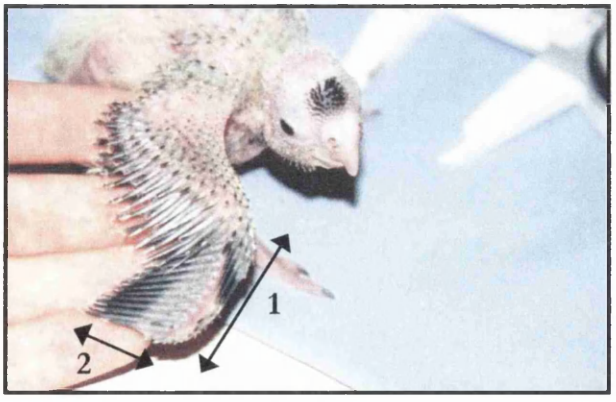


Figure 2.8a Mean clutch mass over all four feeding regimes for Seasons 1, 2 and then combined data over both seasons for first time breeding pairs only.

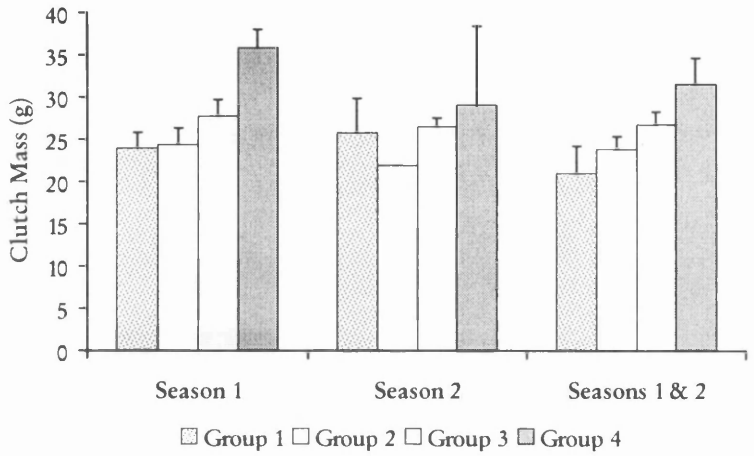


Figure 2.8b Mean egg mass/clutch

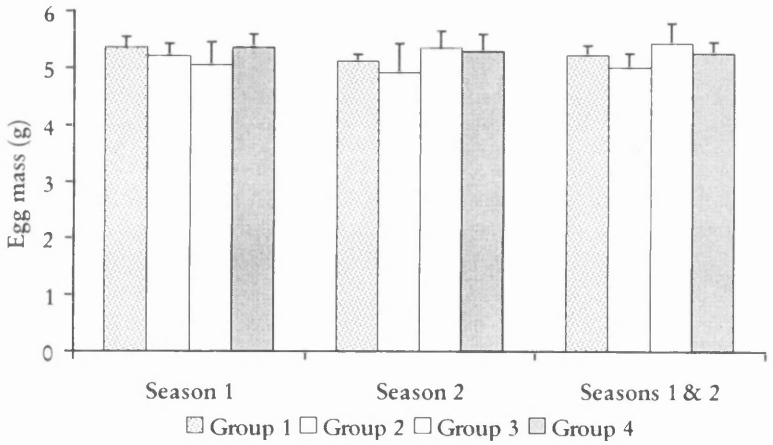


Figure 2.8c Mean clutch size

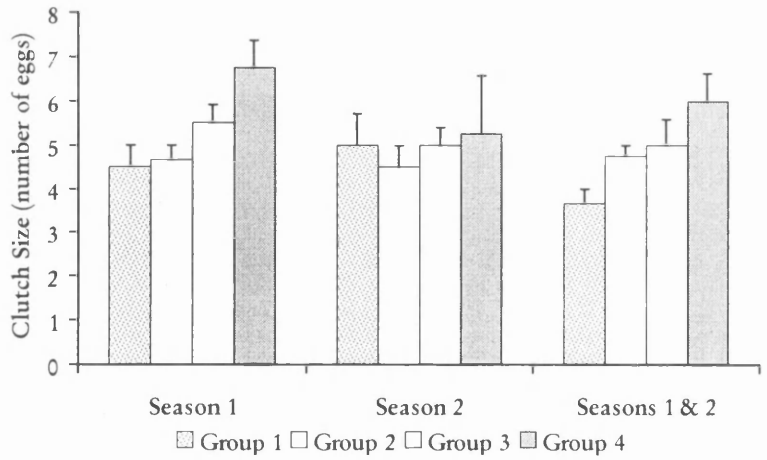


Figure 2.9 Relationship between the number of days a female had been supplemented before she started to lay, and the mass of her first-laid egg.

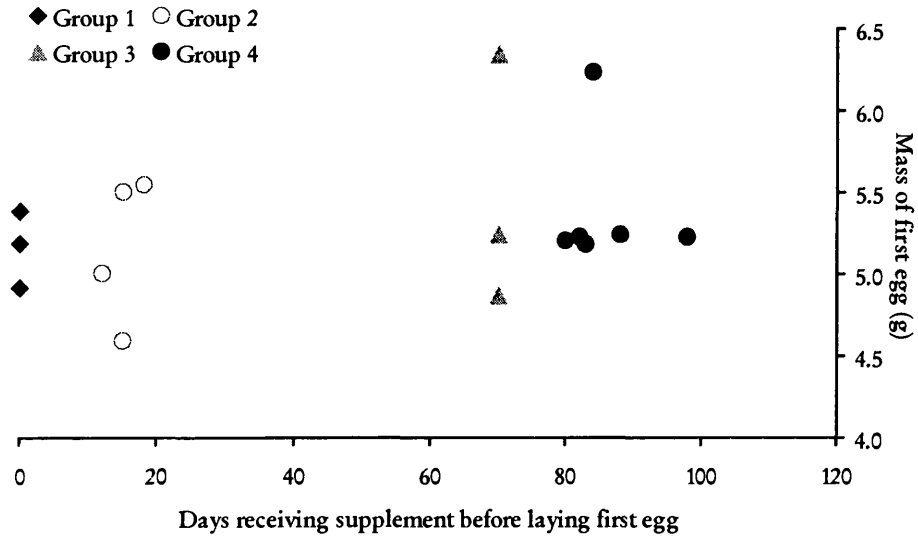


Figure 2.10 Relationship between clutch size and gap days.

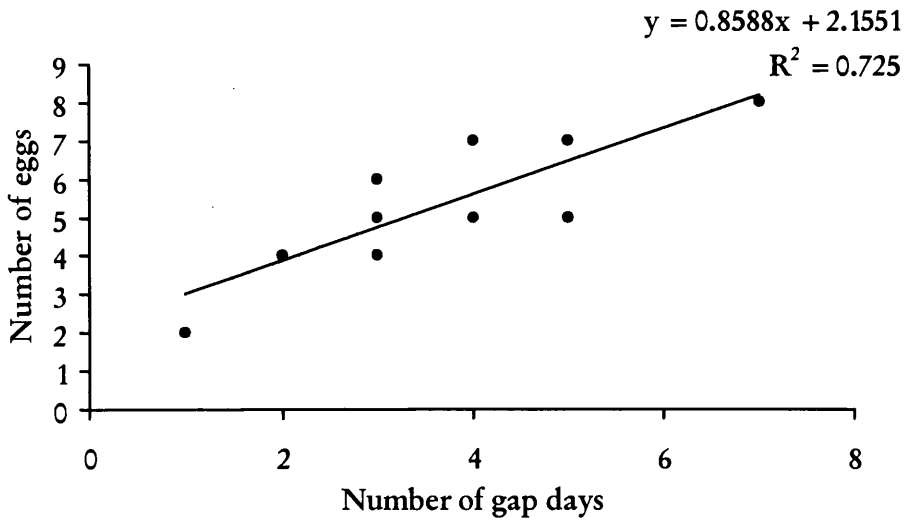


Figure 2.11 Matched-pair data from birds that bred in successive seasons. The legend indicates the diet regime the birds were fed during the ***Pre-laying*** period in Season 1. The birds went on to receive the opposite treatment in the following season, i.e. no supplement birds in Season 1 were fed supplement in Season 2.

Figure 2.11a Mean clutch mass.

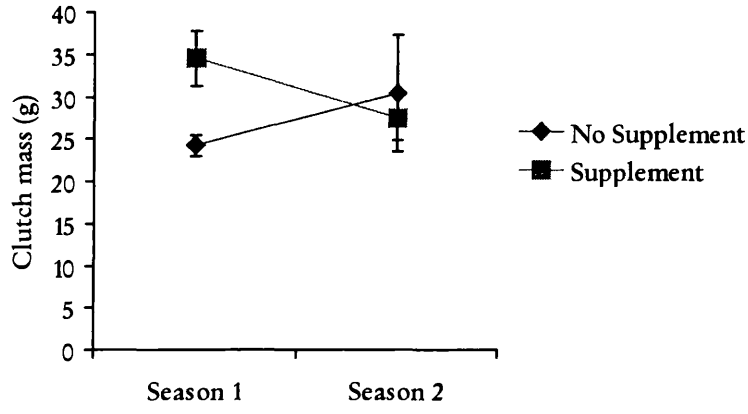


Figure 2.11b Mean clutch size

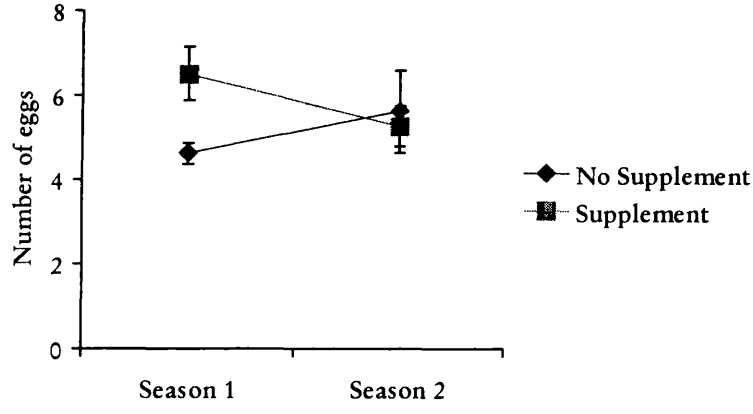


Figure 2.11c Mean egg mass

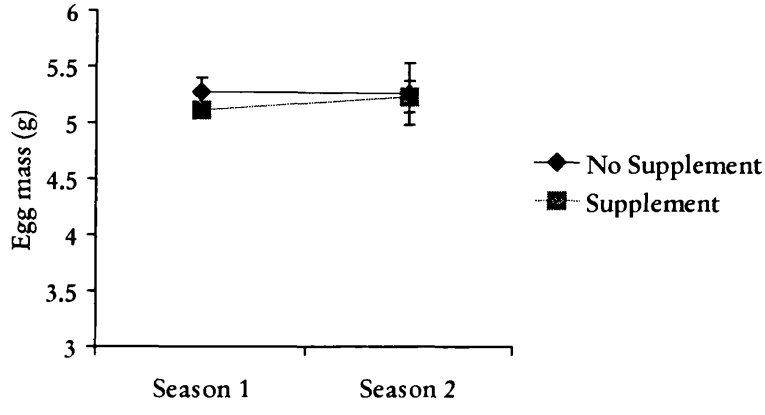


Figure 2.12 Relationship between egg mass and hatch weight.

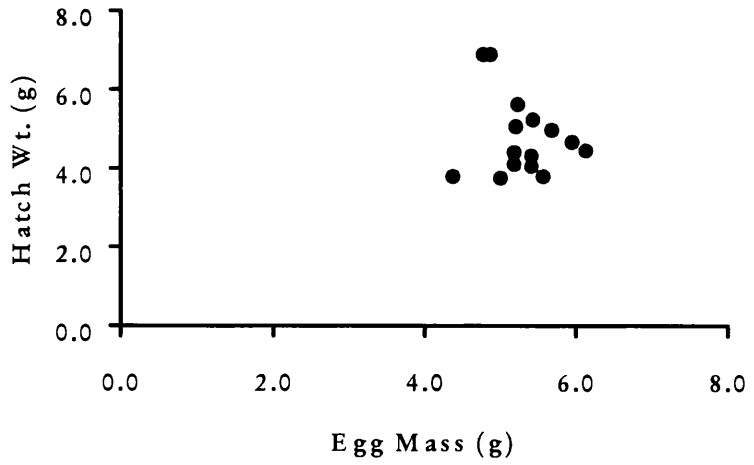


Figure 2.13 Mean increase in chick mass for all four treatments, grouped according to whether the parents were supplemented at any point in the breeding cycle (Supplement: Groups 2-4) or not (No Supplement: Group 1) measured at regular intervals before chicks fledged from the nest at Day 35.

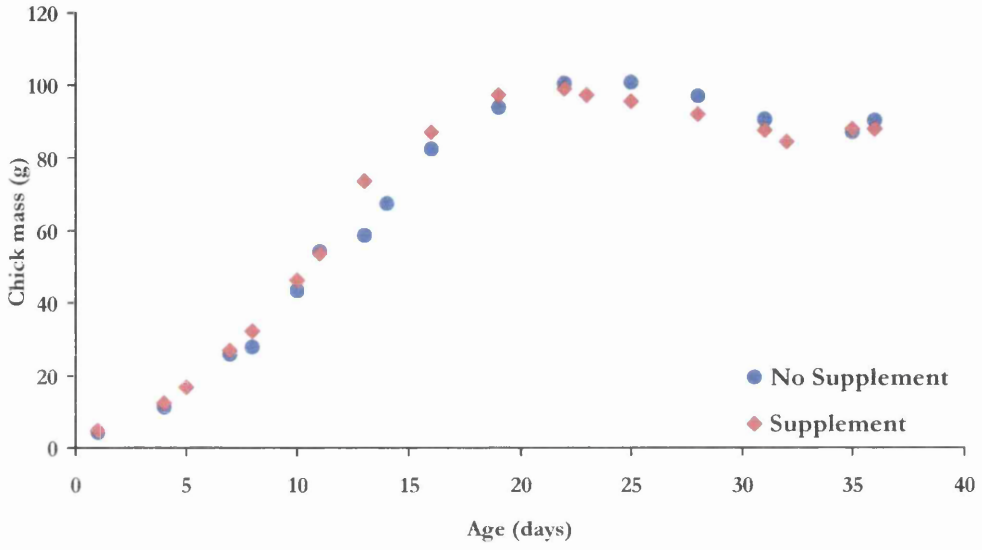


Figure 2.14 Mean increase in chick head-bill length for all four treatments, grouped according to whether the parents were supplemented at any point in the breeding cycle (Supplement: Groups 2-4) or not (No Supplement: Group 1), measured at regular intervals from day of hatching until chicks fledged from the nest at Day 35.

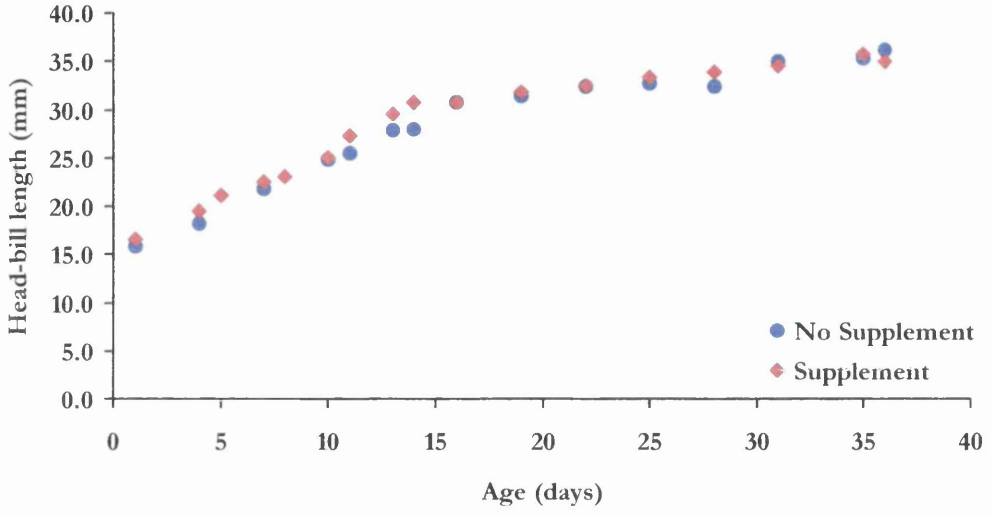


Figure 2.15 Mean increase in chick tarsus length for all four treatments, grouped according to whether the parents were supplemented at any point in the breeding cycle (Supplement: Groups 2-4) or not (No Supplement: Group 1), measured at regular intervals from day of hatching until chicks fledged from the nest at Day 35.

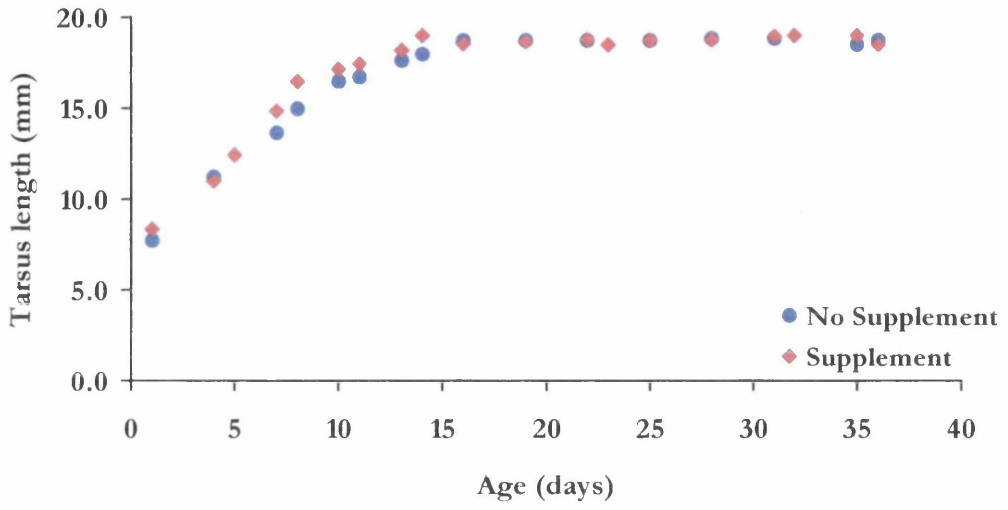


Figure 2.16 Mean increase in chick winglimb length for all four treatments, grouped according to whether the parents were supplemented at any point in the breeding cycle (Supplement: Groups 2-4) or not (No Supplement: Group 1), measured at regular intervals from day of hatching until chicks fledged from the nest at Day 35.

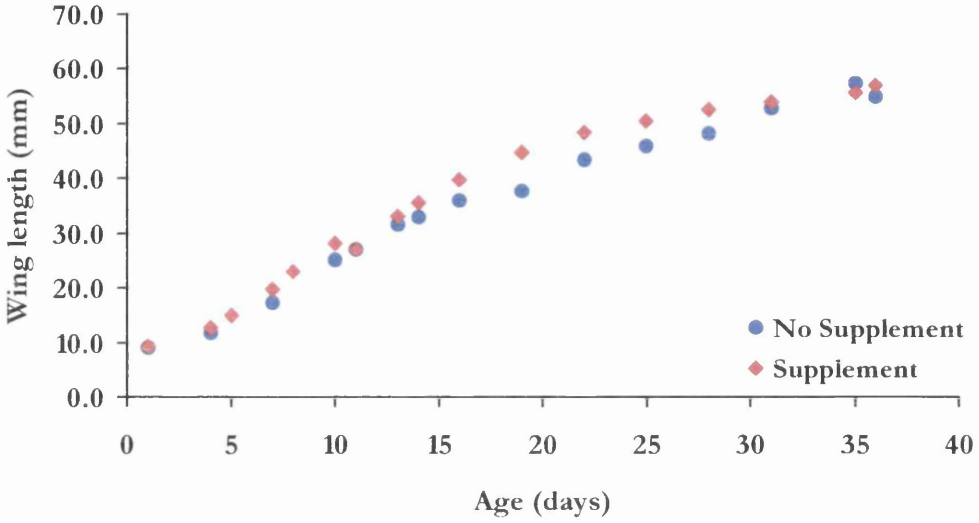
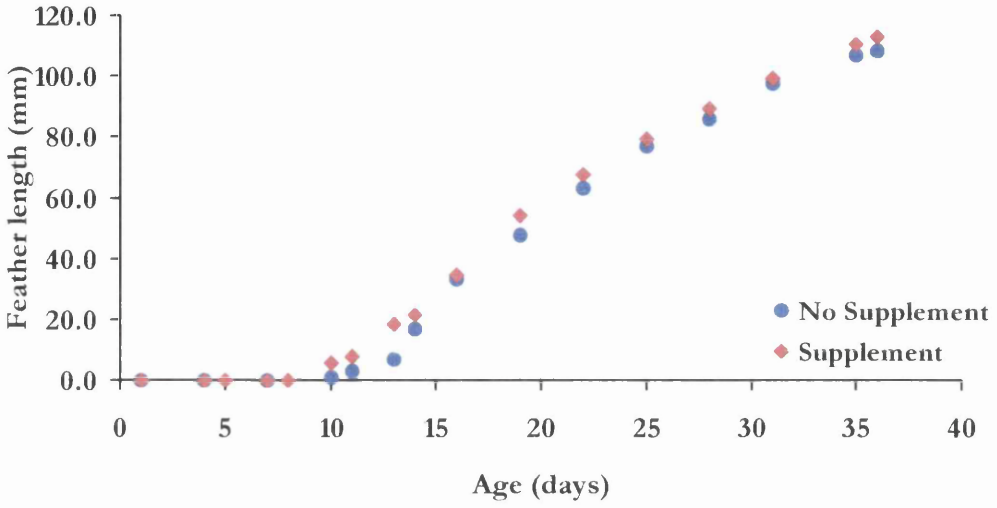


Figure 2.17 Mean increase in chick primary feather length for all four treatments, grouped according to whether the parents were supplemented at any point in the breeding cycle (Supplement: Groups 2-4) or not (No Supplement: Group 1), measured at regular intervals from day of first appearance until chicks fledged from the nest at Day 35.



CHAPTER 3 Interspecific variation in the nutrient composition of avian eggs

INTRODUCTION

A freshly laid avian egg contains all the necessary nutrients and raw materials that will eventually produce a chick. Obvious variation exists in egg size, shape and colour (Figure 3.1), but the components of the egg - the proportion of yolk, albumen and shell - also vary greatly between species (Meyer, 1930; Asmundsen et al., 1943; Romanoff and Romanoff, 1949). As early as 1884, it was noted that the proportion of yolk within an egg varied with respect to the stage of development the chick hatched at along the altricial-precocial spectrum (Tarchanoff, 1884). The eggs of species with precocial development, particularly waterfowl, have large yolks (about 35% of egg weight) compared to the eggs of birds with altricial development (about 20 % egg weight). Nice (1962) categorised eight classes of developmental maturity in birds (see Table 3.1 for a contemporary reorganisation of the diagnostic features by Starck and Ricklefs, 1998) and found a close relationship with yolk size, expressed as a percent of egg weight. At one extreme, the eggs of *Megapodius* have very large yolks (62%) and young *Megapodius* are completely independent of parental care after hatching. At the other end of the precocial-altricial spectrum, eggs of altricial passerines contain 15-27% yolk and remain in the nest for several weeks.

Much of the literature on the composition of bird eggs has concentrated on this variation in yolk content. Because yolk is more dense than albumen and contains a large proportion of lipid, the chemical energy of the contents of fresh eggs varies directly with yolk size. Caloric equivalents of egg contents, obtained primarily by bomb calorimetry and summarised by King (1973) from numerous literature sources, were on average about 1.05 kcal/g for altricial species, 1.6 kcal/g for galliform species and 1.8 kcal/g for anseriform species. However, Schreiber and Lawrence (1976) emphasised that it is more important to determine the chemical constituents of eggs, than to calculate the proportions of yolk and albumen or to measures caloric values directly, since proportions of lipid, protein and water in the yolk also differ between species. A further limitation was that many studies of egg composition were based on reports of individual species. Several authors (Rickleffs, 1974, 1977; Ar and Yom-Tov, 1978; Carey et al., 1980; Sotherland and Rahn, 1987) have attempted to redress this balance by examining differences in egg composition across a

range of species and using the lipid, protein and water contents of the entire egg rather than simply the ratio, or weight, of yolk to albumen.

Albumen composition is relatively constant across species, usually 88-90% water, but yolk composition varies. The water fraction in yolk ranges from about 65% in some altricial species to about 40% in some precocial species. The net result is that the contents of altricial eggs are usually more than 80% water, with an energy density less than 5kJ/g, whereas precocial eggs are usually less than 75% water with an energy density of 6-12kJ/g (Sotherland and Rahn, 1987). Most of the difference in energy density result from difference in water content - the lipid content of dry yolk does not vary greatly between groups, averaging about 58%. As yolk size increases from 20-50% of egg contents, energy density of the dry contents, which averages 29kJ/g increases by only 10%, so energy content can be estimated with reasonable accuracy from the dry mass of the egg contents (Sotherland and Rahn, 1987).

Despite examining yolk and albumen in more detail, most studies have continued to focus predominantly on total lipid or total protein composition of albumen and yolk and we still know very little detail about the nutrient composition of these egg components. The egg composition of the domestic chicken (*Gallus gallus domesticus*) has been exhaustively studied (Romanoff and Romanoff, 1949; Burley and Vadehra, 1989), but few data exist on more specific aspects of egg composition for most wild birds species. Protein is a complex macronutrient consisting of twenty common amino acids, of which nine are considered essential for birds because they lack the specific enzymes for synthesis. Murphy (1994) studied the amino acid composition of bird eggs in order to establish a framework for investigating the role of protein nutrition and metabolism as factors limiting egg production using egg samples from two bird species that produce altricial young, zebra finch (*Taeniopygia guttata*) and pigeon (*Columbia livia*).

Dietary lipids supply energy, fatty acids and pigments. Lipids are the most concentrated energy source a bird can consume and are typically digested and metabolised with high efficiency. Birds are able to synthesise saturated fatty acids *de novo* and to oxidise them up to the ninth carbon inward from the carboxyl end (Δ^9). However they lack the enzymatic capacity to introduce double bonds past the Δ^9, thus they cannot use stearic acid (18 carbons in length) to synthesize linoleic (C18:2$\Delta^{9,12}$) or linolenic acid (C18:3$\Delta^{9,12,15}$). Only

plants have the enzymes capable of inserting Δ^{12} or Δ^{15} double bonds into C_{18} fatty acids and consequently linoleic and linolenic acids are considered essential fatty acids for birds. Descriptions of the fatty acid profiles of yolks from: lesser black-backed gull *Larus fuscus* and pheasant *Phasianus colchicus* (Speake et al, 1996); emperor penguin *Aptenodytes forsteri* (Speake et al., 1999); and a selection of non-domesticated galliforms (Choi et al., 2001), have all been made, simply to expand the knowledge of this topic beyond that of the domestic chicken. Fat-soluble vitamin composition (Surai et al., 2001b), and carotenoid profile (Royle et al., 1999; Surai et al., 2001a) of egg yolks of wild bird species have also been investigated, to determine whether the domestic chicken is an appropriate avian model for the metabolism of these nutrients.

Aims of this chapter

It has been recognised for some time that the initial proportions of yolk and albumen in avian eggs vary considerably between species, most notably with respect to developmental maturity of the chick at hatching, along the altricial-precocial spectrum. Gross composition (total moisture, lipid and protein) is known and documented for many species but, as explained already, detailed composition, comparing amino or fatty acid profiles for example, has only been determined for a very few species. For this study, eggs of 18 bird species, representing 10 avian orders (Table 3.2), a range of dietary habits and many of the eight classes of developmental maturity of chicks at hatching, were collected and analysed. In this chapter I present the gross composition (lipid, protein and water contents), amino acid and essential fatty acid profiles of their eggs. Differences in egg composition are discussed in terms of developmental maturity of chick at hatching, and maternal diet.

MATERIALS & METHODS

Egg collection & preparation

Eggs of 18 bird species, representing 10 avian orders, were collected and analysed (Table 3.2). Eggs of each species were collected under license where applicable (see Table 3.2) and wherever possible, were gathered from more than one nest. Each egg was labelled with a species code and number using permanent ink and weighed immediately after collection. For transportation the egg was placed in a sealed plastic bag with a note of the code and initial egg weight. Eggs were usually processed within 7 days of being laid, having been refrigerated during the interim period. In addition to whole egg weight, maximum

length and width were measured. In the case of fresh eggs, the contents were accessed by first piercing a hole in the air space end of the shell with fine tweezers and gently pulling away shell fragments from the inner membrane. Once there was a sufficiently large hole with a neat edge, I could pour the contents onto a glass dish without puncturing the yolk membrane and mixing the egg contents. Ostrich eggshells required a small drill with a circular blade to cut around the circumference of the shell until I reached the inner membrane. At this point a scalpel was used to puncture the membrane and pour the contents into a large bowl. Once opened, only eggs without any visible evidence of fertilisation (i.e. blood vessels, embryo) were processed.

It was relatively easy to isolate the intact yolk by first rolling the gelatinous 'thick' albumen off the structure then, using fine tweezers, carefully separate it from the rope-like chalazae responsible for suspending the yolk in place within the intact egg (Burley and Vadehra, 1989). The yolk was weighed to 0.1g using a balance. The eggshell and all the fragments removed were rinsed of all albumen using distilled water, patted dry and weighed to 0.1g. They were then air-dried to a constant weight, and it was this dry weight, plus that of the yolk subtracted from the initial whole egg mass, that was used to estimate rather than measure total albumen weight.

Where immediate transportation to Glasgow was not possible, eggs were weighed fresh and then frozen whole at -20°C in sealed plastic bags, again containing a note of the egg's initial weight. The bags minimised loss of albumen material in case the eggshell cracked as the egg contents froze. Frozen eggs were defrosted in glass dishes at room temperature. As the contents thawed it was possible to peel the shell away from the albumen. The albumen, with its higher water content, defrosted much faster than yolk and again it was relatively easy to separate the albumen from the often still frozen yolk. The yolk was allowed to thaw thoroughly before it was weighed, although it never regained its original viscosity. This phenomenon, referred to as yolk gelling, was noted by Moran (1925) on cooling hen's eggs below -6°C. The average freezing point of yolk is -0.65°C. On freezing at temperatures above -6°C, yolk becomes gelatinous, but on rewarming liquefies. Irreversible change therefore occurs below this temperature, but does not occur on supercooling or at very rapid freezing rates (Burley and Vadehra, 1989). Neither of these two latter options were available to me and thus eggs were frozen and stored in -20°C freezers. There does not appear to be any evidence of freezing adversely affecting the

nutrient composition of the egg and indeed all freshly prepared material was frozen until chemical analyses took place. I was advised that repeated freezing and thawing would be detrimental to the egg material (P. Surai, pers. comm), however this process was only necessary twice – in the first instance to separate the egg contents and secondly, to conduct chemical analyses on the samples. As with freshly prepared eggs, the measured dry shell and thawed yolk weights were subtracted from the total egg mass to calculate the weight of albumen.

In both instances, whether eggs prepared either fresh or frozen, samples of albumen and yolk were stored in labelled plastic containers (30ml universal tubes) and stored at -20°C. Egg shells were also labelled and stored at room temperature, although no further analysis was conducted on them.

Chemical analyses of egg material

All chemical analyses of egg composition were conducted by staff at the Central Nutrition Laboratory of Pedigree Masterfoods, the parent company of Waltham Centre for Pet Nutrition. Due to circumstances beyond my control, not all the species of eggs that were processed (as described above and listed in Table 3.2), were subject to chemical analysis. The species not analysed were gentoo penguin (*Pygosdis papua*), bar-headed goose (*Anser indicus*), cockatiel (*Nymphicus hollandicus*) and swift (*Apus apus*). Of those that were, albumen and yolk were assayed separately. The samples were subject to proximate analysis, i.e. measurement of moisture, crude protein (CP), fat and ash, and more detailed analysis to determine total amino acid and essential fatty acid profiles. Moisture and ash were determined by thermogravimetric analysis (Appendix I). Crude protein was determined by the Dumas principle combustion method (using a factor of 6.25 to convert from total nitrogen to CP) (Appendix II).

Total amino acid profile was determined by ion-exchange chromatography as described in Appendix III. Seventeen amino acids were assayed: all the essential amino acids except threonine (which degrades during the process described); - arginine (Arg), iso-leucine (I-Leu), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe), threonine (Thr), and valine (Val). The non-essential amino acids assayed were: alanine (Ala), aspartic acid (Asp), cystine (Cys), glutamic acid (Glu), glycine (Gly), histidine (His), proline (Pro), serine (Ser) and tyrosine (Tyr).

Fat was measured using the Bligh and Dyer extraction method, and the essential fatty acid profile determined thereafter by methylation of the fatty acids, since it renders them more suitable to analysis by gas chromatography, a technique commonly abbreviated to the acronym FAME (Fatty Acid Methyl Esters) analysis. More details are provided in Appendix IV. Where the contents of a single egg were insufficient for a complete set of analyses described above, as in the example of eggs from the crow (*Corvus corone*), two or more eggs were pooled to provide sufficient material and thereafter treated as a single datum point.

RESULTS

Weight of whole eggs and their contents is summarised in Table 3.3 for all the species collected and is comparable with data collated in reviews by Carey et al. (1980) and Sotherland and Rahn (1987). The mean egg weights in this study range over 400-fold, from 3.28g (±0.45) for the swift (*Apus apus*) to 1327.4g (± 74.7) for the ostrich (*Struthio camelus*) eggs. The yolk fraction, sometimes referred to as the yolk/albumen ratio was calculated by dividing the egg contents by the yolk weight. The values range from 0.18 for the shag (*Phalacrocorax aristotelis*) and gannet (*Morus bassana*) to 0.44 and 0.47 for the mute swan (*Cygnus olor*) and black swan (*C. atratus*) respectively. As observed by other authors, there was a significant, positive correlation ($r^2 = 0.74$), between proportion of yolk within the eggs and precocity of chicks at hatching (Figure 3.2).

Proximate composition

Albumen composition is presented in Tables 3.4a and 3.4b. The proximate composition of albumen is quite uniform with respect to water levels and ash (inorganic matter), varying from 85-91% and 0.62-0.91%. respectively. Uncontaminated albumen contains virtually no lipid and both yolk and albumen contain only small amounts of carbohydrates. Although carbohydrates were not determined in this study, Romanoff and Romanoff (1949) reported levels of 0.6-0.8% of hen's albumen wet weight. Inorganic materials (ash), were found to constitute 0.6-0.9% of the albumen fresh weight. After water, the predominant remaining component of albumen is protein, varying from 6.8-10.9%. There was an overall trend for the albumen of altricial eggs to be more dilute, that is to contain more water and less protein.

Yolk composition is presented in Tables 3.5a, 3.5b and 3.6. Yolk contains far less water than albumen and the mean water levels were also more variable, from 44-47% for the anseriforms, to 48 and 51% for the galliforms, up to almost 56% for the altricial species listed. The proportion of yolk protein present tends to be fairly constant, from 12.9-16.1%, and does not show any consistent relationship with the mode of development. Fat comprises between 22-40% of the yolk, with the amount observed in shag eggs being much lower, at just under 14% of the fresh weight. Once more, there does not appear to be a consistent relationship between yolk content and mode of development, given that the examples of anseriform eggs, all in the category 'precocial 2', contain from 26 to 40% fat. Ash levels were found to be 2.0-3.2% of the yolk wet weight.

Amino acid and fatty acid profiles

The total albumen amino acid profiles are presented in Tables 3.4a and 3.4b, grouped according to whether they are classed as essential or not essential. Equivalent data for yolk amino acids are shown in Tables 3.5a and 3.5b. The profiles of all the albumen samples follow a similar pattern to that observed for crude protein, in that the albumen of altricial species appears to contain proportionally less of many of the amino acids, whether essential or non-essential (Tables 3.4a and 3.4b). Yolk amino acid proportions seem far less variable than those observed in albumen, and consistent trends relating to developmental mode are not evident. Figures 3.3a and 3.3b graphically summarise the values measured for each *essential* amino acid in albumen and yolk, using box and whisker plots to show the range, quartiles, minimum and maximum values. Figures 3.4a and 3.4b present equivalent plots for *non-essential* amino acids. From these plots and the coefficient of variation for each of these parameters, methionine, phenylalanine and glutamic acid seem to exhibit the largest range of values in egg albumen, while only methionine appeared as variable in yolk. The variation in proportions observed within each of the named albumen amino acids (methionine, phenylalanine and glutamic acid), showed a consistent and significant correlation with developmental mode – higher levels of each of these amino acids are observed when the hatching maturity is more precocial (Figure 5a, c and d respectively). A similar pattern related to developmental maturity was not observed for methionine in the egg yolk protein (Figure 3.5b).

Essential fatty acid profiles for all the eggs sampled are presented in Table 3.6. There is considerable variability in the proportion of each of them present in the egg yolk, with linolelic acid exhibiting the greatest range, from barely traceable for black swans, to 12% of the yolk lipid for junglefowl (Table 3.6 and Figure 3.6). This range did not appear to be linked with developmental maturity at hatching (Figure 3.7a), but there was some evidence of a relationship with diet, as on the basis of fairly broad dietary categorisation (Figure 3.7b).

As a final comparison I looked at the nutrient profiles of junglefowl, and its domestic counterpart the domestic chicken, and the amino acid values are quite similar (Figure 3.8a and 3.8b), given that the sample size for the domestic egg was one. The essential fatty acid profile however, looks quite different (Figure 3.9), the domestic chicken egg yolk tending to contain far less of each of the fatty acids.

DISCUSSION

When comparing data among studies of interspecific variation in yolk and albumen content, the method used to separate the components must be considered. In previous reviews (Carey et al., 1980; Sotherland and Rahn, 1987), the relative amounts of yolk and albumen in avian eggs have been measured by manual separation of yolk from albumen in fresh eggs, or by boiling the eggs briefly to solidify the contents before separation. Although boiling facilitates the clean separation of egg components, it is at the expense of denaturing egg proteins and lipoproteins, as evidenced by the dramatic hardening of the albumen (Burley and Vadehra, 1989). In a systematic study comparing methods of preparation of European starling eggs, hard-boiling resulted in the loss of about 4% of egg mass, primarily as water from the albumen (Ricklefs, 1982). For my study, all the samples were either prepared after being frozen or having been stored in a refrigerator, the effect of which on egg mass can be considered negligible (Ricklefs, 1982; Burley and Vadehra, 1989), so the values obtained, especially for moisture, are comparable.

Proximate composition

The values for moisture, protein and (where relevant) lipid composition of albumen and yolk, were comparable with previously published data for each species or its closest appropriate relative (Ricklefs, 1977; Carey et al., 1980; Sotherland and Rahn, 1987).

Sotherland and Rahn (1987), found in their review of the egg composition literature that the relative content of yolk does not vary significantly when compared among species in each developmental category. However a precocial egg contains approximately 46% more yolk than a similarly sized altricial egg. It can be concluded that the extra protein and lipid contained in the precocial egg would not only support the metabolic costs of a generally longer incubation period than that of a similarly sized altricial egg; it would also contribute the raw materials for synthesis of feathers, muscles, and other advanced tissues required by precocial hatchlings.

The percentage of albumen in egg content varies inversely with yolk content. The proportion of water in albumen was relatively constant (85-91%) as has been observed by other authors (Ricklefs, 1977; Carey et al., 1980; Sotherland and Rahn, 1987). The proportion of water in yolk was far more variable, from 44-47% for the anseriforms, to 48 and 51% for the galliforms, up to almost 56% for the altricial species listed. Because albumen has a higher relative water content than does yolk, variation in albumen and yolk contents results in inverse changes in water and solid content. Solids comprise about 15% of the egg content in altricial eggs and about 25% of that of precocial eggs (Ricklefs, 1977; Carey et al., 1980; Sotherland and Rahn, 1987). Because albumen contains very little lipid, it follows that, as yolk content increases, a larger amount of protein is carried by the yolk (Sotherland and Rahn, 1987).

Amino acids

Birds laying eggs need dietary amino acids for normal maintenance, growth of the oviduct and accretion of egg proteins (Murphy, 1994). The growth of the oviduct and synthesis of the several yolks are mostly complete before the first egg is laid (Grau, 1984). Consequently , the female's amino acid requirement increases at least a week prior to her first oviposition. In some birds, yolk accretion is extended over several weeks and daily requirements increase very little (Klasing, 1998). In most species, egg albumen is synthesised in the oviduct during a 24 hour period before ovulation. Thus dietary amino acid requirements are especially high on the day preceding each oviposition. Exceptions would be those birds which fully form an egg several days prior to oviposition, e.g. the emu (Klasing, 1998).

Some differences in the amino acids proportions of avian eggs were evident across the range of species sampled. The profiles for both essential and non-essential amino acids in yolk appeared to be highly conserved across species, whereas the values for albumen are less consistent, although this phenomenon did not apply to every amino acid. Methionine, phenylalanine (both essential) and glutamic acid (non-essential) were the most variable and this was shown to be significantly correlated with developmental maturity at hatching. The higher methionine content found in the albumen of precocial species is of particular interest, given that within avian metabolism, as a sulphur-containing amino acid, methionine may be used as a precursor for cystine – an important component of feather production (Murphy, 1994; Klasing, 1998). Murphy (1994), did not observe such wide variation, or strong relationship with developmental mode, but that study comprised eggs from just two altricial species (the zebra finch and pigeon) and compared the results with published data for two precocial birds species (domestic chicken and pintail duck).

Fatty acids

The production of a clutch of eggs requires the deposition of large amounts of yolk lipids, mostly during the several days prior to ovulation. Yolk lipid (and proteins) are synthesised in the liver under the influence of oestrogen and progesterone and are transferred through the blood to the ovarian follicles (Klasing, 1998). In a survey of eggs from 23 species, Christie and Moore (1972) found little variation in the chain lengths of fatty acids among species, but the proportion and distribution of unsaturated carbon-carbon bonds differed considerably. However, it is not clear whether hatchability of the embryo is significantly affected by the relative proportions of saturated and unsaturated fatty acids in the egg.

I found the concentrations of all the essential fatty acids in the species sampled to be highly variable, but of those linolenic acid (C18:3) and arachidonic acid (C20:4) and eicosopentanoic acid (C20:5) were the most conserved and found in the least quantities. The proportions of linoleic acid (C18:2) had the largest range, but this bore no relationship to mode of developmental maturity. Instead, as Christy and Moore (1972) suggest, variations in the chain lengths of fatty acids in the diet are probably the major source of difference among birds of different species.

Further analysis

This chapter was intended to be largely descriptive - the data set is extensive and the analysis presented here is clearly not exhaustive. I chose not to conduct phylogenetic corrections for a number of reasons: although the data presented represent the largest collection of eggs analysed in this manner, it is still a small selection spread thinly across the avian orders. Furthermore, species were selected on ease of access for collecting eggs rather than the best species to make paired comparisons. However, as a data set of total amino acid and essential fatty acid composition for 14 avian species, with quality control ensured by all assays being conducted at the same laboratory using identical methods, it stands as an invaluable resource for future investigations.

Ultimately the laying bird must support the synthesis of nutrients for the egg with those from dietary origin – in advance by depositing protein stores, during egg production directly from the diet each day, or afterwards by repletion of protein reserves (Murphy, 1994). Comparing the chemical profiles of an egg (both yolk and albumen) with profiles for a wide variety of food types will reveal nutritional candidates likely to constrain egg production and improve the provision of adequate diets for captive breeding programmes for endangered bird species.

Table 3.1 Reorganisation of diagnostic features of Nice's developmental classes (from Starck & Ricklefs (1998).

	Plumage (at hatching)	Eyes	Nest Attendance	Parental Care
Precocial 1	Contour feathers			None
Precocial 2			Leave	Brooding
Precocial 3		Open		Led to food
Precocial 4				
Semiprecocial	Down		Nest Area	
Semialtricial 1				Parental feeding
Semialtricial 2		Closed	Stay	
Altricial	None			

Table 3.2 Bird species from which eggs were collected, arranged according to taxonomic order

Order	Common Name	Scientific Name	Source*	Status†
Struthioniformes	Ostrich	*Struthio camelus*	Hangland, Ostrich Farm, UK	Given Food
Sphenisciformes	Gentoo penguin	*Pygoscelis papua*	Edinburgh Zoo, UK	Given Food
	Humboldt penguin	*Spheniscus humboldti*	Chester Zoo, UK	Given Food (F)
Procellariiformes	Fulmar	*Fulmarus glacialis*	Hermaness, Shetland[1]	Wild (F)
Pelecaniformes	Gannet	*Morus bassana*	Ailsa Craig, UK[1]	Wild
	Shag	*Phalacrocorax aristotelis*	Isle of May, UK[1]	Wild
Anseriformes	Black swan	*Cygnus atratus*	Wildfowl & Wetlands Trust, Slimbridge, UK	Given Food (F)
	Mute swan	*Cygnus olor*	Abbotsbury, Dorset, UK[2]	Wild (F)
	Bar-headed goose	*Anser indicus*	Wildfowl & Wetlands Trust, Slimbridge, UK	Given Food (F)
	Mallard	*Anas platyrhynchos*	Wildfowl & Wetlands Trust, Martin Mere, UK	Given Food
	Red crested pochard	*Netta rufina*	Wildfowl & Wetlands Trust, Slimbridge, UK	Given Food (F)
Galliformes	Junglefowl	*Gallus gallus*	Whipsnade Wild Animal Park, UK	Given Food
	Domestic chicken	*Gallus gallus domesticus*	Safeway Supersaver eggs	Given Food
Charadriiformes	Lesser black-backed gull	*Larus fuscus*	South Walney , Cumbria, UK	Wild
	Common Guillemot	*Uria aalge*	Duck Island, Alaska, USA[3]	Wild (F)
Psittaciformes	Cockatiel	*Nymphicus hollandicus*	University of Glasgow, UK	Given Food
Apodiformes	Swift	*Apus apus*	Oxford University Museum Tower, UK[2]	Wild (F)
Passeriformes	Crow	*Corvus corone*	Insh Marshes, Inverness, UK[1]	Wild (F)

*Permission to collect eggs from these locations granted by [1]Scottish Natural Heritage, [2]English Nature and (to Tom van Pelt) [3]U.S. Dept. of Fisheries and Wildlife. † Refers to status of female responsible for laying the eggs, (F) denotes that eggs were frozen after collection.

Table 3.3 Weight of whole egg and contents for all species listed in Table 2, but re-arranged according to developmental maturity of chicks at hatching.

Scientific Name	N	Egg mass (g)	Egg Contents (g)	Yolk (g)	Yolk fraction	Developmental Maturity[1]	Diet Category
Cygnus atratus	6	249.11 ± 14.93	219.43 ± 13.59	103.31 ± 5.75	0.47	Precocial 2	Herbivore
Cygnus olor	4	320.36 ± 15.88	281.98 ± 13.81	124.46 ± 6.21	0.44	Precocial 2	Herbivore
Anser indicus	9	135.49 ± 8.45	123.35 ± 10.29	49.46 ± 3.24	0.40	Precocial 2	Herbivore
Anas platyrhynchos	12	53.10 ± 3.79	50.16 ± 1.46	19.27 ± 1.03	0.38	Precocial 2	Herbivore
Netta rufina	4	49.60 ± 3.70	45.76 ± 4.00	19.80 ± 2.76	0.43	Precocial 2	Omnivore
Struthio camelus	6	1327.4 ± 74.7	1077.2 ± 53.6	326.8 ± 22.7	0.30	Precocial 3	Herbivore
Gallus gallus	6	30.67 ± 2.83	27.49 ± 2.45	12.64 ± 1.49	0.46	Precocial 3	Granivore
Gallus gallus domesticus	1	57.81	53.62	15.55	0.29	Precocial 3	Granivore
Uria aalge	4	119.08 ± 7.06	[2]103.5	[2]37.5	[1]0.36	Semi-precocial	Piscivore
Larus fuscus	15	82.11 ± 7.89	76.48 ± 7.40	20.36 ± 1.54	0.27	Semi-precocial	Omnivore
Fulmarus glacialis	1	94.48	88.26	48.15	0.55	Semi-altricial 2	Piscivore
Pygoscelis papua	1	121.90	107.24	24.39	0.23	Semi-altricial 2	Piscivore
Spheniscus humboldti	4	103.67 ± 11.41	95.32 ± 10.72	25.68 ± 1.79	0.27	Semi-altricial 2	Piscivore
Morus bassana	6	106.42 ± 6.75	96.27 ± 6.21	17.43 ± 0.98	0.18	Semi-altricial 2	Piscivore
Phalacrocorax aristotelis	12	51.66 ± 4.64	46.35 ± 4.15	8.13 ± 0.82	0.18	Altricial	Piscivore
Nymphicus hollandicus	11	5.27 ± 0.33	4.94 ± 0.31	1.30 ± 0.11	0.26	Altricial	Granivore
Apus apus	3	3.28 ± 0.45	3.07 ± 0.46	0.90 ± 0.13	0.29	Altricial	Insectivore
Corvus corone	12	20.85 ± 2.61	19.58 ± 2.51	3.85 ± 0.17	0.20	Altricial	Omnivore

[1]Using the classes described by Nice (1962); [2]Values calculated from egg mass using data published by Sotherland and Rahn, (1987).

Table 3.4a Composition of albumen: proximate values and essential amino acid profiles.

Scientific Name (n)	Water %	Protein %	Ash %	Arg %	I-Leu %	Leu %	Lys %	Met %	Phe %	Thr %	Val %
Cygnus atratus (4)	87.30 ± 0.75	9.68 ± 0.62	0.75 ± 0.76	0.54 ± 0.06	0.54 ± 0.05	0.93 ± 0.10	0.78 ± 0.09	0.63 ± 0.06	0.87 ± 0.10	0.80 ± 0.09	0.81 ± 0.08
Cygnus olor (4)	87.88 ± 0.93	9.73 ± 0.54	0.73 ± 0.10	0.44 ± 0.04	0.48 ± 0.04	0.82 ± 0.06	0.76 ± 0.06	0.49 ± 0.05	0.71 ± 0.06	0.71 ± 0.06	0.71 ± 0.05
Anas platyrhynchos (4)	85.23 ± 0.15	10.91 ± 0.93	0.91 ± 0.07	0.65 ± 0.04	0.56 ± 0.03	1.07 ± 0.06	0.91 ± 0.05	0.65 ± 0.04	0.97 ± 0.05	0.79 ± 0.04	0.86 ± 0.05
Netta rufina (4)	88.38 ± 0.67	7.58 ± 0.73	0.90 ± 0.16	0.46 ± 0.04	0.46 ± 0.05	0.86 ± 0.07	0.73 ± 0.08	0.52 ± 0.03	0.70 ± 0.06	0.60 ± 0.05	0.66 ± 0.06
Uria aalge (4)	87.05 ± 2.81	9.78 ± 1.10	0.75 ± 0.06	0.46 ± 0.11	0.58 ± 0.10	1.07 ± 0.17	0.86 ± 0.15	0.24 ± 0.05	0.68 ± 0.12	0.74 ± 0.13	0.72 ± 0.11
Struthio camelus (4)	89.20 ± 0.86	8.60 ± 0.24	0.73 ± 0.05	0.40 ± 0.05	0.59 ± 0.02	1.04 ± 0.05	0.64 ± 0.17	0.31 ± 0.05	0.55 ± 0.02	0.53 ± 0.04	0.67 ± 0.03
Gallus gallus (6)	90.20 ± 1.81	7.37 ± 1.28	0.85 ± 0.10	0.67 ± 0.15	0.56 ± 0.13	0.90 ± 0.21	0.75 ± 0.18	0.34 ± 0.08	0.65 ± 0.15	0.48 ± 0.12	0.72 ± 0.17
Gallus gallus domesticus (1)	88.30	9.60	0.10	0.56	0.50	0.79	0.63	0.37	0.56	0.44	0.65
Larus fuscus (15)	87.29 ± 0.99	10.18 ± 0.78	0.62 ± 0.13	0.39 ± 0.06	0.53 ± 0.05	1.01 ± 0.09	0.77 ± 0.06	0.47 ± 0.05	0.66 ± 0.07	0.71 ± 0.06	0.73 ± 0.06
Spheniscus humboldti (4)	89.90 ± 0.88	7.38 ± 0.82	0.85 ± 0.06	0.41 ± 0.05	0.47 ± 0.10	0.83 ± 0.17	0.60 ± 0.13	0.24 ± 0.09	0.54 ± 0.01	0.51 ± 0.10	0.58 ± 0.12
Morus bassana (4)	89.70 ± 0.73	7.63 ± 0.83	0.83 ± 0.05	0.31 ± 0.01	0.45 ± 0.03	0.86 ± 0.06	0.57 ± 0.03	0.25 ± 0.03	0.53 ± 0.03	0.49 ± 0.03	0.56 ± 0.03
Phalacrocorax aristotelis (4)	90.13 ± 0.53	7.93 ± 0.94	0.70 ± 0.00	0.28 ± 0.06	0.42 ± 0.08	0.66 ± 0.14	0.52 ± 0.11	0.13 ± 0.02	0.45 ± 0.09	0.37 ± 0.08	0.41 ± 0.09
Corvus corone (4)	91.10 ± 0.00	6.80 ± 0.18	0.90 ± 0.20	0.44 ± 0.03	0.50 ± 0.01	0.66 ± 0.02	0.58 ± 0.01	0.04 ± 0.02	0.42 ± 0.03	0.44 ± 0.20	0.46 ± 0.01
CV	0.28	0.19	0.21	0.24	0.10	0.15	0.17	0.52	0.25	0.22	0.19

Data are presented as mean ± standard deviation; the coefficient of variation (CV) is based on species' means for each nutritional parameter.

Table 3.4b Composition of albumen: non-essential amino acid profiles.

Scientific Name (n)	Ala %	Asp %	Cys %	Glu %	Gly %	His %	Pro %	Ser %	Tyr %
Cygnus atratus (4)	0.49 ± 0.05	1.16 ± 0.14	0.44 ± 0.11	1.62 ± 0.15	0.46 ± 0.05	0.30 ± 0.04	0.46 ± 0.05	0.92 ± 0.09	0.65 ± 0.09
Cygnus olor (4)	0.42 ± 0.03	1.04 ± 0.08	0.35 ± 0.02	1.42 ± 0.10	0.41 ± 0.04	0.25 ± 0.02	0.39 ± 0.03	0.81 ± 0.07	0.53 ± 0.04
Anas platyrhynchos (4)	0.59 ± 0.04	1.20 ± 0.06	0.36 ± 0.04	1.82 ± 0.11	0.51 ± 0.06	0.31 ± 0.03	0.48 ± 0.03	1.05 ± 0.11	0.62 ± 0.03
Netta rufina (4)	0.48 ± 0.05	0.97 ± 0.09	0.32 ± 0.03	1.42 ± 0.15	0.38 ± 0.03	0.23 ± 0.02	0.39 ± 0.03	0.76 ± 0.08	0.43 ± 0.04
Uria aalge (4)	0.51 ± 0.10	1.05 ± 0.19	0.33 ± 0.16	1.62 ± 0.27	0.39 ± 0.08	0.22 ± 0.04	0.49 ± 0.08	0.79 ± 0.16	0.55 ± 0.10
Struthio camelus (4)	0.50 ± 0.05	0.88 ± 0.03	0.22 ± 0.01	1.52 ± 0.05	0.33 ± 0.01	0.23 ± 0.01	0.43 ± 0.04	0.76 ± 0.04	0.53 ± 0.02
Gallus gallus (6)	0.61 ± 0.14	1.11 ± 0.27	0.27 ± 0.06	1.43 ± 0.32	0.39 ± 0.09	0.27 ± 0.06	0.38 ± 0.09	0.73 ± 0.16	0.44 ± 0.11
Gallus gallus domesticus (1)	0.55	0.99	0.20	1.30	0.35	0.25	0.33	0.64	0.40
Larus fuscus (15)	0.49 ± 0.03	0.98 ± 0.07	0.29 ± 0.08	1.55 ± 0.12	0.40 ± 0.04	0.26 ± 0.03	0.50 ± 0.04	0.78 ± 0.08	0.56 ± 0.05
Spheniscus humboldti (4)	0.37 ± 0.07	0.81 ± 0.17	0.24 ± 0.07	1.20 ± 0.25	0.31 ± 0.06	0.21 ± 0.04	0.38 ± 0.07	0.61 ± 0.12	0.47 ± 0.09
Morus bassana (4)	0.38 ± 0.02	0.77 ± 0.04	0.19 ± 0.02	1.15 ± 0.06	0.29 ± 0.02	0.20 ± 0.02	0.40 ± 0.02	0.55 ± 0.03	0.39 ± 0.03
Phalacrocorax aristotelis (4)	0.31 ± 0.07	0.61 ± 0.13	0.16 ± 0.01	1.03 ± 0.22	0.20 ± 0.05	0.18 ± 0.04	0.30 ± 0.07	0.45 ± 0.09	0.30 ± 0.06
Corvus corone (4)	0.30 ± 0.01	0.72 ± 0.01	0.24 ± 0.02	1.14 ± 0.04	0.28 ± 0.01	0.17 ± 0.01	0.30 ± 0.01	0.67 ± 0.04	0.39 ± 0.03
CV	0.22	0.19	0.29	0.16	0.22	0.18	0.15	0.21	0.21

Data are presented as mean ± standard deviation; the coefficient of variation (CV) is based on species' means for each nutritional parameter.

Table 3.5a Composition of yolk: proximate values and essential amino acid profiles.

Scientific Name (n)	Water %	Protein %	Fat %	Ash %	Arg %	Iso-L %	Leu %	Lys %	Met %	Phe %	Thr %	Val %
Cygnus atratus (4)	44.73 ± 1.42	15.48 ± 0.33	26.03 ± 2.04	3.00 ± 0.08	1.11 ± 0.06	0.81 ± 0.01	1.27 ± 0.02	1.11 ± 0.08	0.41 ± 0.02	0.72 ± 0.05	0.77 ± 0.06	0.87 ± 0.02
Cygnus olor (4)	45.25 ± 0.99	12.93 ± 0.94	36.9 ± 0.34	2.33 ± 0.15	1.17 ± 0.03	0.83 ± 0.02	1.33 ± 0.04	1.19 ± 0.03	0.20 ± 0.03	0.70 ± 0.03	0.80 ± 0.02	0.86 ± 0.02
Anas platyrhyndos (4)	45.09 ± 0.85	15.21 ± 0.42	39.29 ± 3.06	3.16 ± 0.17	1.18 ± 0.07	0.78 ± 0.02	1.31 ± 0.04	1.17 ± 0.07	0.28 ± 0.04	0.71 ± 0.04	0.80 ± 0.03	0.85 ± 0.02
Netta rufina (4)	47.50 ± 0.80	13.0 ± 0.83	40.40 ± 3.20	2.50 ± 2.00	1.02 ± 0.13	0.71 ± 0.07	1.16 ± 0.12	1.05 ± 0.12	0.29 ± 0.06	0.63 ± 0.07	0.72 ± 0.08	0.77 ± 0.08
Uria aalge (4)	51.88 ± 3.86	15.55 ± 1.12	31.10 ± 2.00	2.03 ± 0.29	1.09 ± 0.18	0.78 ± 0.05	1.24 ± 0.08	1.21 ± 0.11	0.14 ± 0.03	0.69 ± 0.04	0.75 ± 0.05	0.87 ± 0.05
Struthio camelus (4)	46.38 ± 0.75	16.08 ± 0.17	25.00 ± 1.81	2.90 ± 0.18	1.23 ± 0.05	0.85 ± 0.01	1.28 ± 0.02	1.32 ± 0.02	0.17 ± 0.06	0.70 ± 0.10	0.79 ± 0.02	0.85 ± 0.02
Gallus gallus (6)	48.12 ± 1.97	15.52 ± 0.89	35.37 ± 1.40	3.12 ± 0.23	1.16 ± 0.04	0.78 ± 0.01	1.30 ± 0.03	1.11 ± 0.04	0.26 ± 0.05	0.69 ± 0.01	0.76 ± 0.02	0.86 ± 0.01
Gallus gallus domesticus (1)	51.3	17.3	30.2	2.8	1.18	0.84	1.37	1.23	0.41	0.70	0.81	0.93
Larus fuscus (15)	48.59 ± 3.10	15.75 ± 0.95	32.95 ± 2.97	2.41 ± 0.28	1.09 ± 0.10	0.82 ± 0.04	1.26 ± 0.07	1.23 ± 0.09	0.33 ± 0.09	0.67 ± 0.03	0.77 ± 0.05	0.88 ± 0.05
Spheniscus humboldti (4)	50.78 ± 1.11	15.58 ± 0.45	25.85 ± 1.06	2.35 ± 0.17	1.09 ± 0.16	0.81 ± 0.04	1.23 ± 0.06	1.19 ± 0.11	0.31 ± 0.13	0.67 ± 0.01	0.74 ± 0.03	0.83 ± 0.04
Morus bassana (4)	55.43 ± 2.32	13.63 ± 1.01	22.75 ± 1.34	2.15 ± 0.21	0.93 ± 0.07	0.71 ± 0.06	1.11 ± 0.08	1.09 ± 0.10	0.24 ± 0.11	0.57 ± 0.05	0.64 ± 0.05	0.77 ± 0.06
Phalacrocorax aristotelis (4)	55.10 ± 0.65	14.45 ± 0.42	13.90 ± 1.08	1.98 ± 0.10	0.94 ± 0.05	0.72 ± 0.04	1.15 ± 0.06	1.18 ± 0.12	0.26 ± 0.14	0.62 ± 0.02	0.70 ± 0.03	0.80 ± 0.04
Corvus corone (4)	55.83 ± 2.10	13.37 ± 0.71	32.35 ± 2.32	2.60 ± 0.29	0.98 ± 0.07	0.70 ± 0.04	1.07 ± 0.05	1.09 ± 0.06	0.03 ± 0.03	0.61 ± 0.03	0.58 ± 0.04	0.71 ± 0.04
CV	0.08	0.09	0.18	0.16	0.09	0.07	0.07	0.06	0.41	0.07	0.09	0.07

Data are presented as mean ± standard deviation; the coefficient of variation (CV) is based on species' means for each nutritional parameter.

Table 3.5b Composition of yolk: non-essential amino acid profiles.

Scientific Name (n)	Ala %	Asp %	Cys %	Glu %	Gly %	His %	Pro %	Ser %	Tyr %
Cygnus atratus (4)	0.74 ± 0.01	1.30 ± 0.03	0.34 ± 0.04	1.82 ± 0.05	0.46 ± 0.02	0.45 ± 0.03	0.61 ± 0.02	1.10 ± 0.08	0.73 ± 0.06
Cygnus olor (4)	0.75 ± 0.01	1.34 ± 0.04	0.28 ± 0.02	1.87 ± 0.04	0.49 ± 0.01	0.42 ± 0.02	0.63 ± 0.01	1.15 ± 0.04	0.70 ± 0.02
Anas platyrhynchos (4)	0.74 ± 0.02	1.35 ± 0.03	0.25 ± 0.01	1.89 ± 0.05	0.49 ± 0.04	0.46 ± 0.03	0.59 ± 0.01	1.24 ± 0.09	0.72 ± 0.05
Netta rufina (4)	0.66 ± 0.06	1.21 ± 0.12	0.27 ± 0.06	1.66 ± 0.18	0.43 ± 0.05	0.40 ± 0.05	0.56 ± 0.07	1.05 ± 0.14	0.61 ± 0.06
Uria aalge (4)	0.77 ± 0.06	1.23 ± 0.11	0.28 ± 0.01	1.78 ± 0.13	0.46 ± 0.03	0.40 ± 0.03	0.61 ± 0.04	1.16 ± 0.10	0.66 ± 0.04
Struthio camelus (4)	0.75 ± 0.01	1.23 ± 0.02	0.31 ± 0.01	1.84 ± 0.03	0.49 ± 0.02	0.44 ± 0.01	0.65 ± 0.01	1.24 ± 0.04	0.71 ± 0.02
Gallus gallus (6)	0.74 ± 0.01	1.39 ± 0.03	0.31 ± 0.01	1.82 ± 0.04	0.47 ± 0.1	0.41 ± 0.01	0.61 ± 0.01	1.24 ± 0.04	0.65 ± 0.02
Gallus gallus domesticus (1)	0.80	1.53	0.53	2.05	0.47	0.43	0.64	1.32	0.73
Larus fuscus (15)	0.81 ± 0.05	1.34 ± 0.10	0.31 ± 0.12	1.90 ± 0.11	0.48 ± 0.03	0.41 ± 0.02	0.62 ± 0.04	1.21 ± 0.09	0.70 ± 0.04
Spheniscus humboldti (4)	0.71 ± 0.04	1.27 ± 0.08	0.30 ± 0.04	1.74 ± 0.09	0.46 ± 0.02	0.41 ± 0.02	0.61 ± 0.03	1.15 ± 0.06	0.72 ± 0.01
Morus bassana (4)	0.68 ± 0.05	1.16 ± 0.10	0.19 ± 0.02	1.60 ± 0.13	0.39 ± 0.02	0.35 ± 0.03	0.55 ± 0.04	1.00 ± 0.09	0.59 ± 0.06
Phalacrocorax aristotelis (4)	0.73 ± 0.05	1.22 ± 0.10	0.26 ± 0.01	1.69 ± 0.09	0.41 ± 0.02	0.37 ± 0.01	0.57 ± 0.04	1.16 ± 0.05	0.67 ± 0.02
Corvus corone (4)	0.63 ± 0.03	1.12 ± 0.07	0.23 ± 0.01	1.63 ± 0.11	0.40 ± 0.03	0.33 ± 0.03	0.50 ± 0.03	1.20 ± 0.09	0.56 ± 0.03
CV	0.06	0.08	0.14	0.07	0.08	0.09	0.07	0.07	0.08

Data are presented as mean ± standard deviation; the coefficient of variation (CV) is based on species' means for each nutritional parameter.

Table 3.6 Composition of yolk: essential fatty acid profile.

Scientific Name (n)	Linoleic (C18: 2) %	Linolenic (C18:3) %	Arachidonic (C20:4) %	Eicospentanoic (C20:5) %	Docoshexanoic (C20:6) %
Cygnus atratus (4)	0.01 ± -	0.04 ± 0.01	1.40 ± 1.60	0.01 ± -	0.29 ± 0.18
Cygnus olor (4)	3.87 ± 0.67	1.34 ± 1.23	1.66 ± 0.07	0.63 ± 0.21	1.17 ± 0.59
Anas platyrhynchos (4)	5.09 ± 0.41	0.42 ± 0.06	1.59 ± 0.12	0.34 ± 0.07	1.51 ± 0.13
Netta rufina (4)	2.69 ± 0.27	0.19 ± 0.03	2.53 ± 0.24	0.08 ± -	0.56 ± 0.12
Uria aalge (4)	1.19 ± 0.17	0.40 ± 0.08	1.15 ± 0.61	3.51 ± 0.28	3.96 ± 0.31
Struthio camelus (4)	8.36 ± 4.23	5.55 ± 1.89	1.66 ± 0.35	0.07 ± 0.12	1.01 ± 0.17
Gallus gallus (6)	12.21 ± 3.14	0.44 ± 0.26	1.98 ± 0.13	0.01 ± -	1.33 ± 0.20
Gallus gallus domesticus (1)					
Larus fuscus (15)	1.20 ± 0.29	0.09 ± 0.05	0.85 ± 0.23	0.06 ± 0.04	0.50 ± 0.14
Spheniscus humboldti (4)	1.96 ± 0.15	0.68 ± 0.07	2.44 ± 0.32	1.74 ± 1.28	3.64 ± 0.28
Morus bassana (4)	0.10 ± -	0.02 ± 0.02	3.64 ± 0.55	0.23 ± 0.15	5.43 ± 0.24
Phalacrocorax aristotelis (4)	1.31 ± 0.09	0.10 ± 0.09	2.14 ± 0.10	3.47 ± 0.26	6.90 ± 0.43
Corvus corone (4)	7.84 ± 0.25	1.35 ± 0.38	3.24 ± 0.29	0.47 ± 0.11	2.23 ± 0.20

Data are presented as mean ± standard deviation.

Figure 3.1 A selection of shells from wild bird eggs sampled.

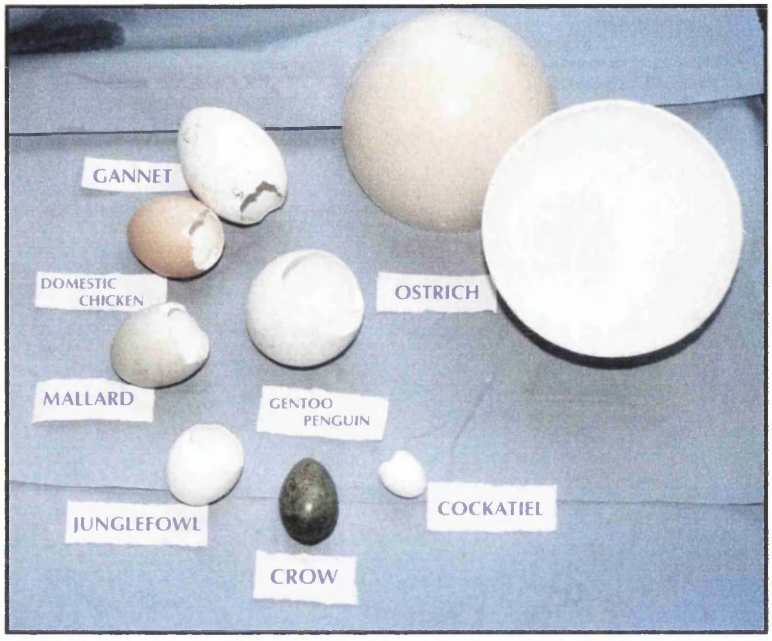


Figure 3.2 Relationship between variation in yolk fraction and difference in developmental maturity between species of eggs sampled.

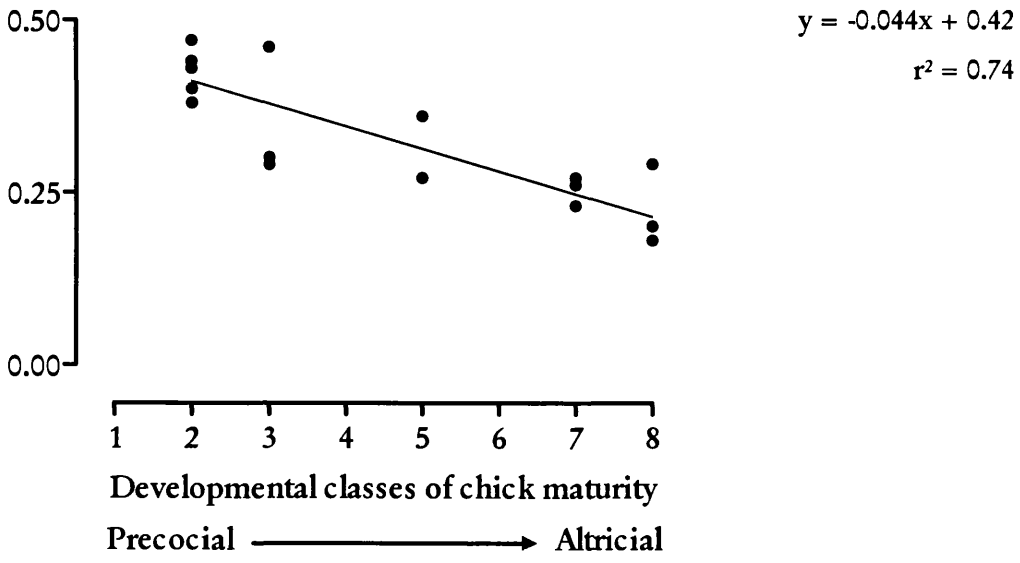
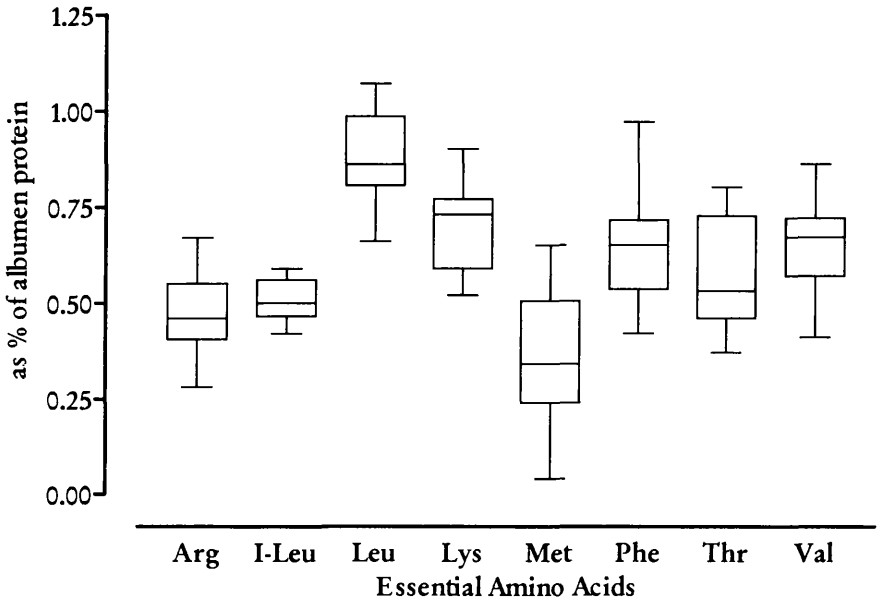


Figure 3.3 Essential amino acid profiles for wild bird eggs sampled. Box and whisker plots shows range and quartiles: the box extends from the 25th percentile to the 75th percentile, with a line at the median (the 50th percentile); the whiskers extend above and below the box to show the maximum and minimum values.

a. Range of essential amino acid concentrations from all species of wild bird egg **albumen**



b. Range of essential amino acid concentrations from all species of wild bird egg **yolk**

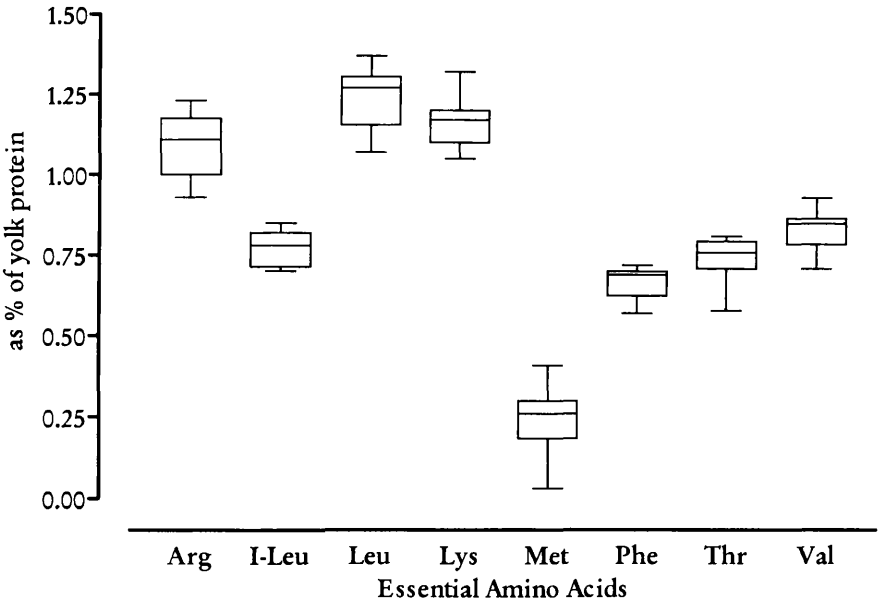
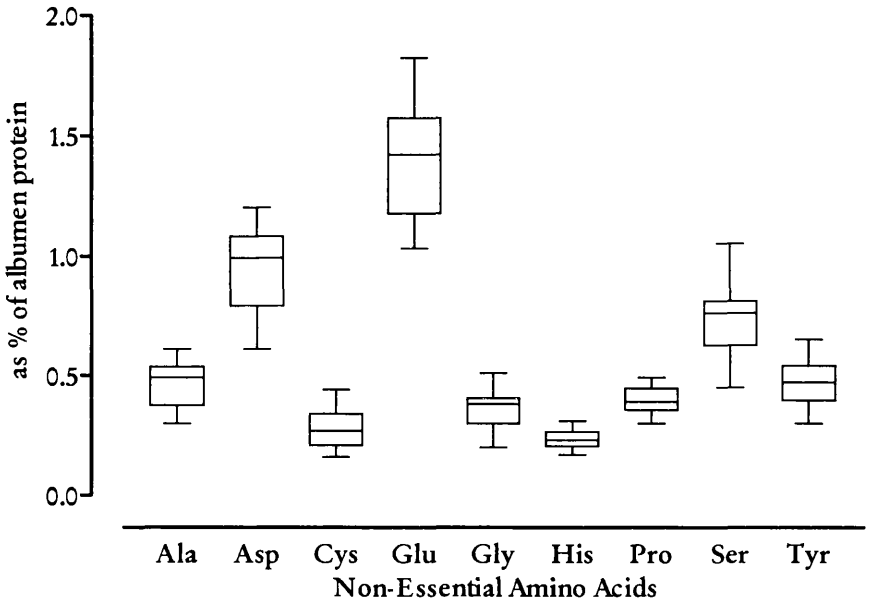


Figure 3.4 Non-essential amino acid profiles for wild bird eggs sampled. As before: the box extends from the 25th percentile to the 75th percentile, with a line at the median (the 50th percentile); the whiskers extend above and below the box to show the maximum and minimum values.

a. Range of non-essential amino acid concentrations from all species of wild bird egg **albumen**



b. Range of non-essential amino acid concentrations from all species of wild bird egg **yolk**

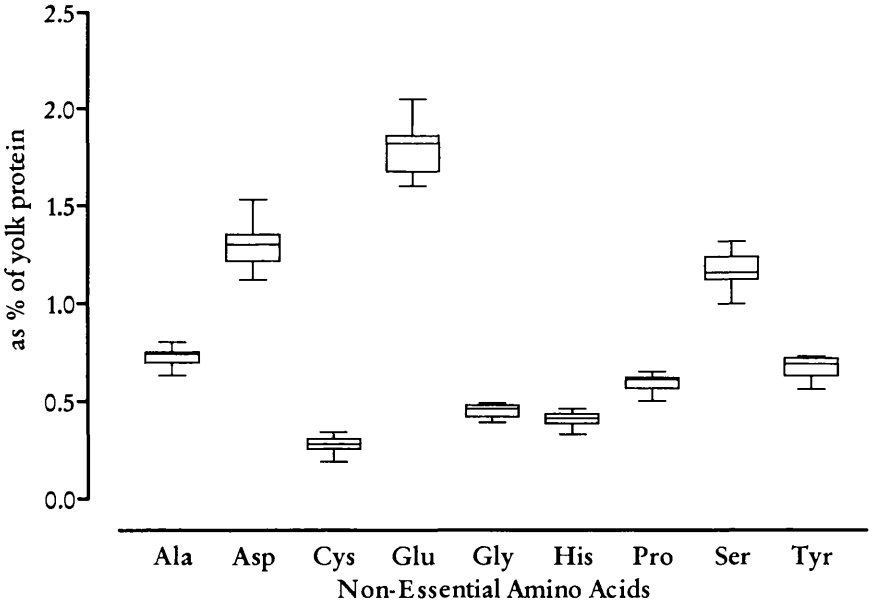
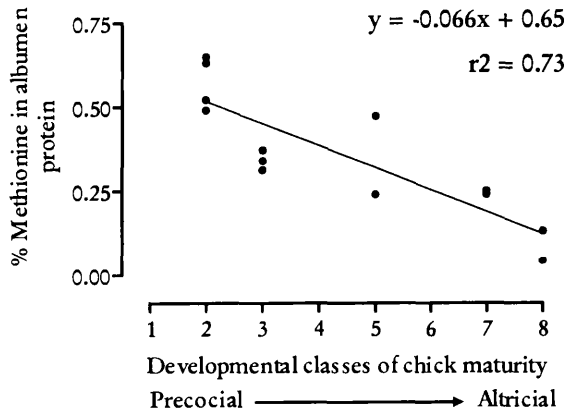
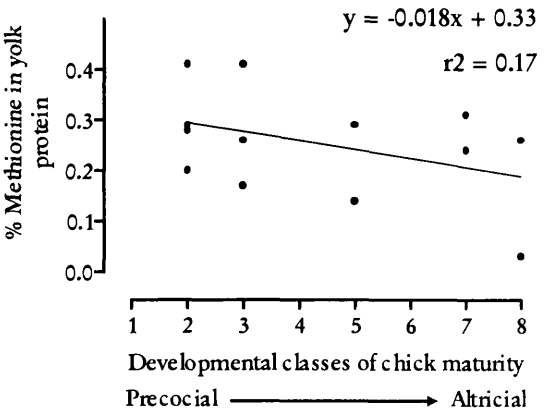


Figure 3.5 Relationship between variation in selected amino acid concentrations and differences in developmental maturity between species of eggs sampled:

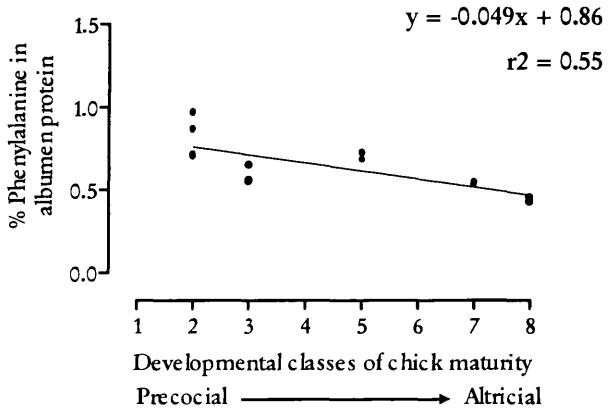
a. Methionine in albumen protein



b. Methionine in yolk protein



c. Phenylalanine in albumen protein



d. Glutamic acid in albumen

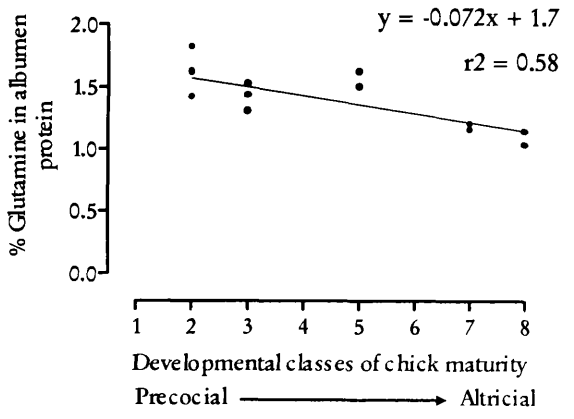


Figure 3.6 Essential fatty acid profiles for wild bird egg yolks sampled. As before: the box extends from the 25th percentile to the 75th percentile, with a line at the median (the 50th percentile); the whiskers extend above and below the box to show the maximum and minimum values.

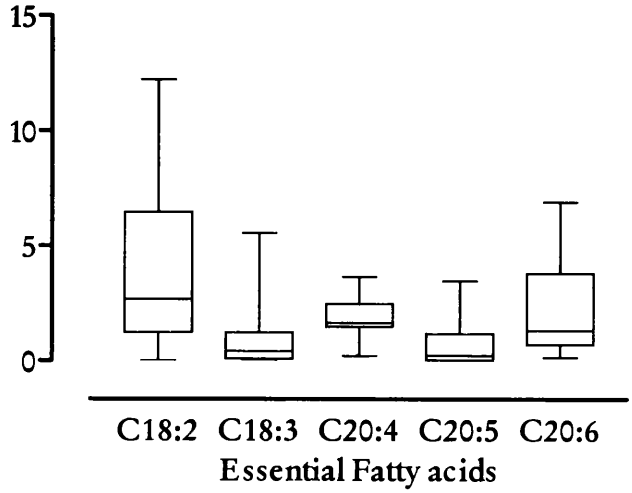
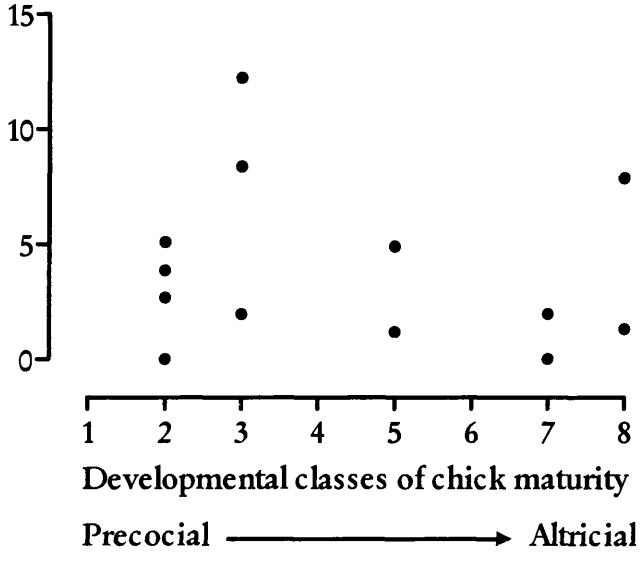


Figure 3.7 Proportion of linoleic acid in egg yolk lipid in relation to a) differences in developmental maturity between species of eggs sampled or, b) differences in diet.

a. Linoleic acid and developmental maturity



b. Linoleic acid and diet

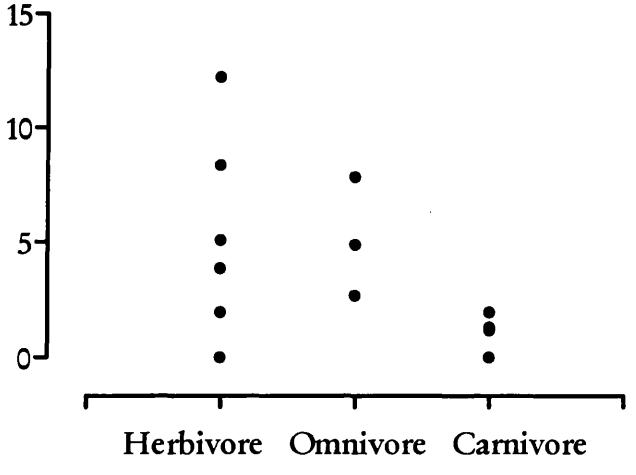


Figure 3.8 Comparison of amino acid profiles for junglefowl and domestic chicken egg albumen (a) and yolk (b).

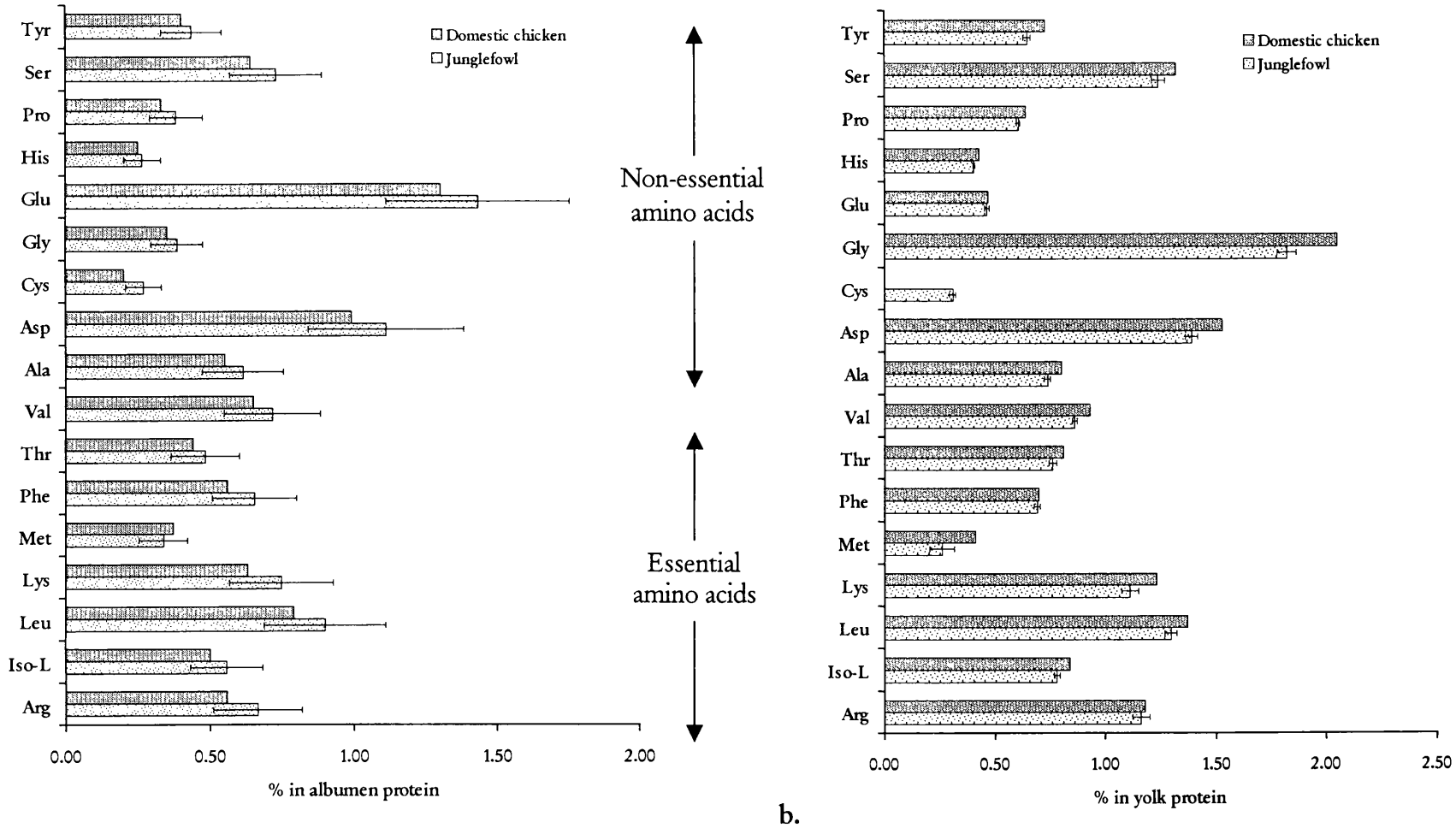
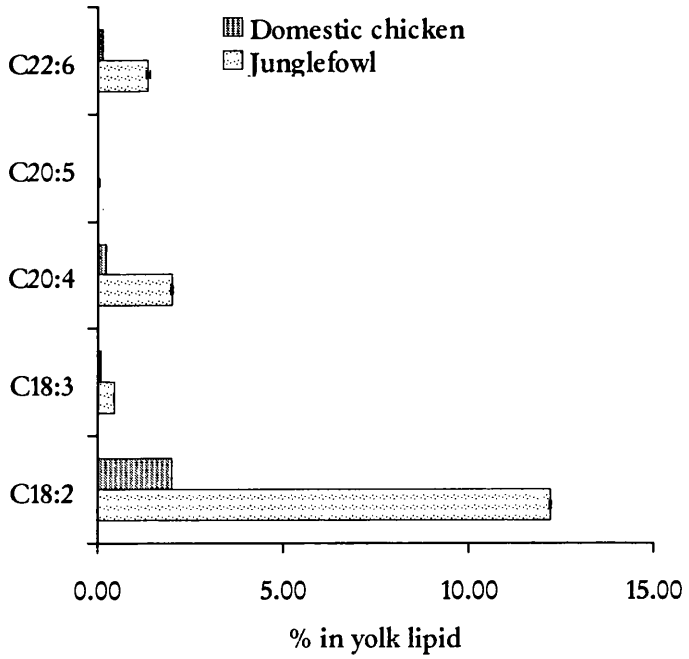


Figure 3.9 Comparison of essential fatty acid profiles for junglefowl and domestic chicken egg yolks.



CHAPTER 4 A comparison of methods to measure immunoglobulin Y levels in the egg yolk of lesser black-backed gulls (*Larus fuscus*).

INTRODUCTION

The new-born young of many species are relatively immuno-incompetent and initially reliant on immunity acquired passively from parents. In mammals there is some transfer of maternal antibody across the placenta during development, or via colostrum milk after birth. Birds however, must deposit all the components required to confer protection at the time of egg-laying. It has long been recognised that hens can transmit parasite resistance to chicks (Klemperer, 1893) and transfer of immunoglobulin from hen serum to yolk was confirmed by studies using radio-labelled molecules (Patterson et al., 1962). Transmission of immunity in other avian species has been demonstrated by inoculation of the female with a novel pathogen and challenging her offspring (Brambell, 1970).

More detailed studies of the avian humoral immune system, principally involving the domestic chicken egg, have described the presence of three major classes of antibodies, proteins of the immunoglobulin (Ig) class. The three groups identified are considered to be analogous to human IgA, IgG and IgM (Rose and Orlans, 1974; Rose and Orlans, 1981). In humans, IgA is mainly found in mucous secretions, defending external body surfaces. IgG is generally considered the most abundant immunoglobulin component of internal body fluids, combating micro-organisms and their toxins. IgM is produced early in the immune response and is largely confined to the blood stream where it acts as an effective agglutinator against bacterial antigens (Roitt et al., 1998). Returning to the chicken egg, the majority of immunoglobulin is IgG, often referred to as IgY (Leslie and Clem, 1969) since it is located in the yolk, with trace amounts of IgA detectable in both the yolk and albumen and IgM, trace levels only, also detected in the albumen (Rose and Orlans, 1974; Rose and Orlans, 1981).

In a laying hen, serum IgG averages 15 mg/ml and endocytosis of IgG by developing oocytes removes 100-200 mg from maternal circulation daily, accounting for 10-20% of the hen's steady-state level. At hatch, the serum IgG of the chick is comparable to, but always slightly less than, that of the hen (Kowalczyk, 1985). Maternally derived immunoglobulin

has been observed in the yolks of turkey (Goudswaard et al., 1978) and pigeon eggs (Goudswaard et al., 1977). Transfer of specific antibody activity has been documented in anseriform (Toth and Norcross, 1981), psittaciform (Lung et al., 1996), charadriform (Gasparini et al., 2001) and passerine species (Hasselquist et al., 2001). While the egg remains the only route for antibody transmission in most bird species, transfer of antibody via feeding of crop milk has been observed in pigeons. In one experiment, both parents were shown to transfer radio-labelled IgA from their serum to crop milk and the labelled IgA was detected in the squab's serum (Goudswaard et al., 1979). IgG has also been detected in crop milk (Engberg et al., 1992), although the authors caution against comparisons with mammalian colostrum since considerable resorption of crop milk immunoglobulins (both IgA and IgG) does not take place.

The ability of females to confer protection against specific pathogens to their offspring has been of interest in commercial systems producing poultry, duck and quail, largely as a means of reducing transmission of common diseases under intensive rearing conditions (Jeurissen and Janse, 1998). Little is known from wild birds, although stimulated by the increasing number of expensive birds kept as pets, Baghian et al., (1999) describe the production of cockatiel anti-Ig, and maternal antibody transfer has been measured in macaws (Lung et al., 1996). Both studies aim to provide diagnostic tools for vets to develop vaccines and preventative health programs for psittacines.

Aims of this chapter

Apart from these domesticated and companion bird species described, there have been no studies on the variation in maternal transfer of immunocompetence in any wild bird species. I investigated this for the lesser black-backed gull (*Larus fuscus*). In order to do this I needed to compare the effectiveness of various assays for the identification and quantification of immunoglobulins in egg yolk of *Larus fuscus*, and this chapter describes the methods employed. There are three main aims to this chapter. Firstly commercial reagents are available to measure immunoglobulin in chicken egg yolks, and I investigated their effectiveness for gulls. Anti-gull egg yolk immunoglobulin (α-gull IgY) reagents were produced for this study and the assay results compared to determine if they produced a better result than that from the chicken reagents. Secondly, there has been comparatively little work done on immunoglobulin levels in wild bird eggs. I investigated which of the two quantitative methods used (single radial gel immunodiffusion and enzyme-linked

immunosorbent assay) was best for analysing such material. Finally, I considered how levels of IgY in wild bird yolks compared with known values for poultry.

MATERIALS AND METHODS

Isolation of gull yolk IgY and production of sheep anti-gull immunoglobulin IgY

Eggs from lesser-blacked back gulls were collected under permit from South Walney Nature Reserve, Walney Island, UK, as part of a larger field study. Eggs from six different birds were used to provide a pool of yolk material for isolation of immunoglobulin Y. The purification and subsequent production of anti-gull immunoglobulin Y (α-gull IgY) raised in sheep, was carried out by Diagnostics Scotland, adapting the method of Polson and von Wechmar, (1980). Throughout this paper, the term 'first bleed' refers to α-gull IgY collected from the sheep after the first injection of antigen (gull IgY) – the primary immune response. The 'second bleed' is α-gull IgY collected from the sheep after a second challenge with the antigen. This secondary response is known to be characterised by more rapid and more abundant production of antibody (Roitt et al., 1998) and therefore, unless stated otherwise, this more concentrated α-gull IgY is used in most of the assays.

The concentration of antibody in the purified gull IgY was determined in two ways. Diagnostics Scotland estimated the concentration by measuring the optical density (O.D.) of IgY at 280 nm, the nearest round number for the peak absorption of protein. This value was then compared to a standard curve drawn up from the O.D. values of dilutions of a standard IgG solution obtained from Sigma. The O.D. of a 1:10 dilution of purified gull IgY was measured at 280 nm and when compared to the standard this gave a value equivalent to 0.120 x 10 (dilution factor) and therefore a concentration of 1.20 mg/ml (L. Bence, pers. comm.). For the second method I measured the O.D. of a 1:6 dilution of purified gull IgY in phosphate-buffered saline. However, to estimate the IgY concentration I applied an extinction factor of 1.4 (the absorbance value of 1 mg/ml standard protein solution) to the O.D value, taking the dilution factor into account and the result was 1.42 mg/ml. These values of 1.2 and 1.42 mg/ml are comparable and the variation is acceptable given the slightly different methodologies employed (M. Kennedy, pers. comm.).

Since all the IgY found in the gull eggs was derived from maternal serum, blood samples were also collected (under Home Office license) from 6 different gulls, centrifuged and the serum pooled for use in assays as a comparison with yolk samples. It would be expected that IgY concentration would be higher in sera than in yolk and I therefore included sera in my analysis in case higher levels of IgY than that present in yolk were necessary to get a positive reaction.

Double radial gel immunodiffusion (Ouchterlony Test)

This assay was performed using both α-chicken and α-gull reagents. In double radial immunodiffusion, agar gels are poured onto slides and allowed to set; wells are then punched in the gel and the test solutions of antigen (Ag) and antibody (Ab) are added. The solutions diffuse out and where Ag and Ab meet they bind to each other, cross-link and precipitate, leaving a line of precipitation. The precipitin bands can be visualised by washing the gel to remove soluble proteins then staining the precipitin arcs with a protein stain (Roitt et al., 1998). The gel was 0.9 % agarose (Sigma, St. Louis, USA) in barbiturate buffer on 7.5 x 2.5 cm glass slides. A punch was used to cut six wells arranged in a hexagonal shape around a further central well. The wells were all 2 mm in diameter and spaced approximately 4 mm from each other (see Figure 4.1a). The α-IgY was added (from either chicken or gull) to the central well and the sera and yolk samples were then pipetted into the surrounding wells. This hexagonal arrangement was duplicated on each slide.

During preliminary trials using undiluted yolk, the high lipid content of the yolk tended to mask any diffusion taking place. To overcome this effect, yolk samples from gull and chicken eggs were first diluted 1:5 in phosphate-buffered saline, then centrifuged for 5 minutes and a sample was taken from the supernatant. Since IgY is derived from maternal serum, both yolk and serum samples were tested with anti-serum samples from gulls and chickens and 10 µl/well of either serum or yolk sample was added in each instance. Double radial immunodiffusion is purely a qualitative assay and was performed to observe reaction between test material and the different sources of anti-IgY. Although it is not normally used to quantify the strength of the reaction, the intensity of the bands does give a rough indication of the qualitative strength of the antibody-antigen cross-reactivity.

Immunoelectrophoresis

This assay was performed using only α-gull reagents. During immunoelectrophoresis, antigens are separated on the basis of their charge before being visualised by precipitation. Samples are placed in two circular wells cut into an agar plate and antigens are separated by placing an electric charge across the gel. The gel's pH is chosen so that positively charged proteins move to the negative electrode (cathode) and negatively charged proteins to the positive (anode). After this separation a trough is cut between the wells and filled with the antibody, which is left to diffuse overnight (see Figure 4.1b). The antigens and the antibody form precipitin arcs which can be visualised by staining in the same manner as described for double radial immunodiffusion.

Immunoelectrophoresis was run on 1 % agarose gel made in barbitone buffer on 7.5 x 2.5 cm glass slides. Yolk samples were prepared as described above and 5 μl/well was used for both yolk and serum samples. The gel was run at 15 μA for 45 minutes, after which the resolved proteins were exposed to 100 μl of sheep anti-gull immunoglobulin IgY placed in the trough. This qualitative test again simply demonstrates a reaction between test material and anti-gull immunoglobulin IgY, but improves on the information available from a double radial immunodiffusion assay because the sample proteins have first been separated in the agar gel, thereby differentiating between albumin and the various globulin proteins that may be present. Most serum protein molecules move towards the anode, but IgG characteristically migrates towards the cathode. Since all the IgY found in the gull eggs was derived from maternal serum and more specifically IgG, I would expect IgY present in the yolk samples to behave in the same manner as IgG and migrate to the cathode.

Single radial immunodiffusion

This assay was performed using only α-gull reagents. In single radial immunodiffusion, antibody is incorporated into the agar gels before they are poured onto slides and allowed to set; wells are then punched in the gel and the test solutions of antigen (Ag) are added. The solutions diffuse out and, where Ag and Ab meet, they bind to each other, cross-link and precipitate leaving a ring of precipitation around the well. The diameter of the precipitate rings is related to the antigen concentration in the wells. A calibration curve can be constructed from the diameter of rings around wells containing known quantities of antigen, which the test sample concentrations can be directly related to. The precipitin

rings can be also visualised by the same techniques used in the Ouchterlony test; washing the gel to remove soluble proteins, then staining the precipitin arcs with a protein stain (Roitt et al., 1998). This quantitative assay was performed by incorporating α-gull IgY into the gel, to determine whether measurable differences could be detected between test yolk samples.

The gel was 0.9 % agar in barbiturate buffer primed with 0.07 % of sheep anti-gull immunoglobulin IgY on 7.5 x 2.5 cm glass slides. Six wells, 2 mm in diameter were punched into the agar (see Figure 4.1c). Four of the wells were filled with serial dilutions of gull sera while the remaining two wells were filled with test yolk samples. The two test yolk samples on each slide comprised of a sample each from the first and last laid egg of a single clutch, to determine whether the assay was sufficiently sensitive to detect within-clutch differences in immunoglobulin concentration. First and last eggs from two clutches were assayed in triplicate (i.e. each slide in Figure 1c was repeated three times for two pairs of yolk samples). The yolk was prepared as described previously and 10 μl of gull yolk or serum was added to each well. Unstained slides were photographed using a digital camera capture system and a slide graticule was also photographed at the same focal depth. The images are then imported into Scion Software (Scion Corporation, USA) permitting more accurate measurement of ring diameter. The 'line' tool is used to select a straight line of known length on the graticule image that is then set as the scale, converting the pixels to a 'known' distance. I used a distance of 1 mm to set the scale. Thereafter, the software converted the ring diameter of the test samples photographed on each slide from pixels to millimetres. The diameter of each ring was measured at its widest point both horizontally and vertically three times, the mean of all these measurements being the final value used to determine antigen concentration.

Enzyme-linked immunosorbent assay (ELISA)

This assay was performed using both α-chicken and α-gull reagents. The techniques of immunoassay using labelled reagents for detecting antigens and antibodies are extremely sensitive and economical in the use of reagents. Antigen in saline is incubated on a plastic plate and small quantities become absorbed onto the plastic surface. Free antigen is washed away and the plate may then be blocked with excess of an irrelevant protein to prevent any subsequent non-specific binding of proteins. Test antibody is added, which binds to the antigen and unbound proteins are washed away. The antibody is detected by a ligand, a

molecule which can detect the antibody and is covalently coupled to an enzyme such as peroxidase. The bound ligand is visualised by the addition of chromogen, a colourless substrate which is acted on by the enzyme portion of the ligand to produce a coloured end-product (Roitt et al., 1998). Intensity of colour (and therefore concentration) is measured electronically by a plate reader.

Enzyme-linked immunosorbent assay was run on test yolk samples to observe cross-reactivity of either α-chicken or α-gull immunoglobulin with yolk samples, and to compare relative concentrations of immunoglobulins in yolk samples using only the first and last eggs from individual clutches. In the protocol described below, adapted from Hudson and Hay (1989), the addition of an enzyme is a two-step process; the test antibody is first sandwiched between antigen on the plate and biotinylated antigen. Streptavidin phosphatase (the enzyme) is then added and the avidin and biotin bind to each other. The methods for measuring the α-chicken and α-gull immunoglobulin were very similar, but minor differences are noted at the relevant stages. Furthermore, the assays were conducted over an 18 month period (November 1998 – May 2000) and yolks were not tested using both α-chicken and α-gull immunoglobulin.

A 96 well microtitre plate was coated with either α-chicken or α-gull immunoglobulin IgY (0.002μg/ml) in bicarbonate/carbonate buffer (pH 9.6) adding 50μl/well, overnight at 4°C. Thereafter all incubations described take place at 37°C. The plate wells were then washed and drained twice with PBS tween using a Nunc Immunowash 12 (this method and reagent applies for all washes). After draining, the wells were blocked with 10% foetal calf serum in PBS (FCS/PBS), adding 200μl/well for at least 1 hour. The test yolk samples were diluted in FCS/PBS as described before; the optimum dilution for α-chicken immunoglobulin was 1/5 and for α-gull immunoglobulin 1/10. The plates were washed twice more and this time after draining dilutions of standard for calibration and test yolk samples were added, using 50μl of material per well and the plate was then incubated for at least 2 hours. After incubation plates were washed and drained four times before the addition of the conjugate, biotinylated α-chicken or α-gull immunoglobulin IgY at 1:1000 dilution, 50μl/well for at least 1 hour. Plates were washed and drained six times and 100μl/well of streptavidin phosphatase at 1:1000 dilution was added before plates were further incubated for 1 hour. Plates were finally washed eight times and TMB substrate

was added after draining, at 100µl/well just before the plate was read. The reactions in the plate were read by Biolinx 2.20 ELISA reading programme on a MRX Microplate reader (Dynatech Laboratories, Virginia) with a 630-nm filter.

RESULTS

Double radial immunodiffusion - Ouchterlony test

In double radial immunodiffusion, some degree of cross-reaction was always observed between IgY antibody of both species and all the test antigens. However it was very obvious that the precipitin bands varied in strength and were more pronounced when the antibody and test antigen were homologous, originating from the same bird species (see Figure 4.2a for an example). Therefore although double radial immunodiffusion is not a quantitative assay it was possible to score the bands as weak or strong examples of cross-reactivity and the results are summarised in Table 4.1. Double precipitin bands were observed in reactions between the sheep anti-gull immunoglobulin IgY and the test gull samples (Figure 4.2a) and one explanation might be that there were two isoforms of the IgG protein present in the samples.

Following these results, only α-gull IgY was used in the immunoelectrophoresis and single radial immunodiffusion test, because this obviously produced a far stronger cross-reactivity with gull IgY than the α-chicken IgY reagents.

Immunoelectrophoresis

Both yolk and serum samples were separated by electrophoresis in an agar gel before being exposed to sheep anti-gull immunoglobulin IgY in order to resolve the proteins responsible for the double precipitin bands observed in the gel immunodiffusion (Figure 4.2b). The yolk sample still showed two bands at the same point on the gel, again suggestive of two isoforms of immunoglobulin with similar weights and charges. However the precipitin arcs from the serum sample suggest it contains just one molecule analogous to that observed in yolk, and a separate protein that also reacted with the sheep anti-gull immunoglobulin IgY.

Single radial immunodiffusion

Using the single radial gel immunodiffusion technique, the sheep anti-gull immunoglobulin IgY clearly reacted with all test samples to creating precipitin rings and was found to be sensitive to the range of serum dilutions used to calibrate each slide (see Figure 4.2c). The ring diameter around wells containing dilutions of serum was measured in millimetres, the values squared and then plotted on a linear scale against the serum dilution value to create a calibration curve. Squaring the diameter values resulted in the calibration being a straight line (see Figure 4.3), from which a linear regression was used to derive the relative IgY titre of unknown test samples of yolk presented in Table 4.2 (serum dilution = - 0.189 + 0.0108 ring diameter^2, $r^2 = 0.974$, $p < 0.001$).

Enzyme-linked immunosorbent assay (ELISA)

In both the chicken and gull systems, optical density readings were plotted against the dilution factor of the homologous serum samples, to create a calibration curve of serum antibody titre. Log transforming both axes resulted in the curve being a straight line to which a linear regression could be fitted and this was used to derive the relative immunoglobulin titre of unknown test samples of yolk. The ELISA assay using chicken reagents was sufficiently sensitive to detect variation in antibody titres between yolk samples collected from different female gulls and between eggs laid by the same female in a single clutch. A t-test for matched pairs determined this variation to border on significance ($t = 2.27$, $p = 0.053$, $n = 9$). Based on the weak cross-reactivity observed between chicken and gull reagents in the double radial immunodiffusion assay, I was concerned that the binding affinity for chicken-gull IgY may be weak and not reflect an consistent measure of immunoglobulin in gull egg yolk. Furthermore, the titre was related to chicken IgG as a standard and gull antibody titre would only be expressed in these terms.

Using gull reagents in an ELISA I was able to detect significantly different variation in antibody titres between yolk samples collected from different female gulls, and between eggs laid by the same female in a single clutch ($t = 2.36$, $p < 0.05$, $n = 22$; t-test for matched pairs). Yolk immunoglobulin values were expressed in relation to gull serum immunoglobulin titre and the cross-reactivity appeared to be amplified (Figure 4.4) although the titres cannot be compared directly since in each instance homologous serum was used.. That is, chicken serum was used as a control with anti-chicken immunoglobulin and gull serum with anti-gull immunoglobulin.

DISCUSSION

This is the first study to investigate total immunoglobulins levels in eggs of wild bird species and I needed to establish which methods were appropriate to detect immunoglobulin in the lipid-rich environment of egg yolk.

Commercial reagents are available to measure immunoglobulin in chicken egg yolks because chicken egg yolk represents an alternative source of polyclonal antibodies. Polyclonal antibodies are extensively used in research and industry and have traditionally been raised in mammals such as rabbits, mice, rats, horses, goats and sheep. Large mammals are expensive to maintain yet small animals yield only small quantities of antibody. A laying hen can produce five to six eggs per week and as such can yield egg antibodies, often referred to as IgY (Leslie and Clem, 1969), equivalent to 90 - 100 ml of serum or 180 - 200 ml of whole blood. By comparison, an immunised rabbit yields 20 ml whole blood/week when repeatedly bled and the procedure is far more time-consuming and invasive (Larsson et al., 1993). The main obstacle to overcome has been the high lipid content of yolk, leading to various methods for purification of egg yolk immunoglobulin (Jensenius et al., 1981; Jensenius and Koch, 1993; Polson et al., 1985; Deignan et al., 2000).

Although commercially available anti-chicken immunoglobulin from Sigma did bind to, and detect immunoglobulin in the gull yolk samples, it was clear that the cross-reactivity between chicken and gull egg yolk immunoglobulins was weak (Figure 4.2a). The anti-gull egg yolk immunoglobulin produced by Diagnostics Scotland for this study resulted in much stronger cross-reactivity, using both qualitative (double radial immunodiffusion assay, Figure 4.2a) and quantitative (enzyme-linked immunosorbent assay, Figure 4.4) methods of detection. The anti-chicken immunoglobulin from Sigma is a highly purified reagent, raised against chicken serum IgG (rather than chicken egg yolk immunoglobulins) , both of which are factors that would negatively influence its cross-reactivity with gull yolk material. The anti-gull reagents were derived from gull yolk directly, rather than serum and since they were not intended for commercial use were not subject to as much purification, thereby enhancing the potential for cross-reactivity with my test gull yolk samples.

Both of the two quantitative methods used to measure immunoglobulin titre, single radial gel immunodiffusion and enzyme-linked immunosorbent assay, were sufficiently sensitive

to pick up titre variation in yolk samples collected from the same female and between those collected from different females. The single radial gel immunodiffusion assay is simpler, although without the digital camera image capture system it would have been difficult to measure the precipitin ring diameters accurately. However this technique has been used by a colleague to detect significant changes in yolk immunoglobulin titre in relation to their laying sequence from normal (3-egg) clutches of *Larus fuscus*, (Blount et al., in press).

I was interested in a method of measuring variation in yolk immunoglobulins from gulls which had laid extended clutches of eggs, looking at patterns of immunoglobulin deposition throughout the entire laying sequence. Under experimental conditions gulls can lay three times the normal clutch size of three eggs (Nager et al., 2000). Although I measured differences in immunoglobulin titre between first and last eggs using single radial immunodiffusion assay (Table 4.2), I was concerned that it may not be sufficiently sensitive to variation throughout the laying sequence. The enzyme-linked immunosorbent assay appeared to amplify the signal produced by cross-reactivity of anti-gull yolk immunoglobulin with test yolk samples and it is for this reason that I chose to use enzyme-linked immunosorbent assay to measure variation in yolk immunoglobulins across extended laying sequences.

It is important to emphasise that, although both quantitative assays, single radial gel immunodiffusion and the enzyme-linked immunosorbent assay provide only a relative measure of gull yolk immunoglobulin titre, which in the context of this chapter was related to gull serum immunoglobulin titre. Calculating absolute levels of immunoglobulin in the test samples was beyond the scope of this study. I was interested in measuring variation between eggs and relative titre values were sufficient for this purpose.

Table 4.1 Cross-reactivity of IgY antibody derived from either chicken or gull material against test antigens using the double radial gel immunodiffusion assay. Based on n=2 observations, (✓) indicates weak cross-reaction, (✓✓) indicates strong cross-reaction.

	Test Antigen			
	Chicken		Gull	
ANTIBODY	serum	Yolk	serum	yolk
α-chicken	✓✓	✓✓	✓	✓
α-gull (1st bleed)	✓	✓	✓✓	✓✓
α-gull (2nd bleed)	✓	✓	✓✓	✓✓

Table 4.2 Precipitin ring diameters of unknown yolk samples and their serum IgG titre equivalence, based on n=3 observations.

Yolk samples (clutch size)	Ring Diameter[2]	Serum IgG titre equivalence
Nest A (9 eggs)		
First laid egg	36.85 ± 0.86	0.21 ± 0.01
Last laid egg	28.81 ± 1.11	0.12 ± 0.01
Nest B (5 eggs)		
First laid egg	34.75 ± 2.02	0.19 ± 0.02
Last laid egg	28.88 ± 1.42	0.12 ± 0.02

Figure 4.1 (a). Arrangement of wells and antigen (Ag) and antibody (Ab) samples, used in double radial immunodiffusion assay.

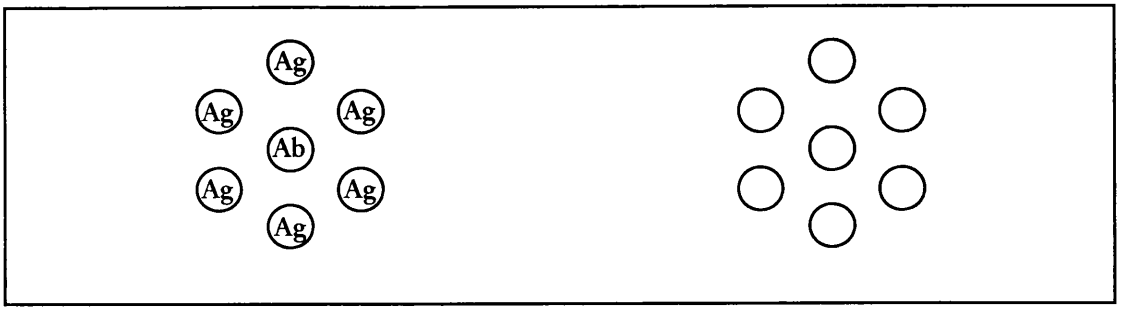


Figure 4.1 (b). Arrangement of wells containing antibody (Ab) samples and trough of antigen (Ag) used in immunoelectrophoresis assay.

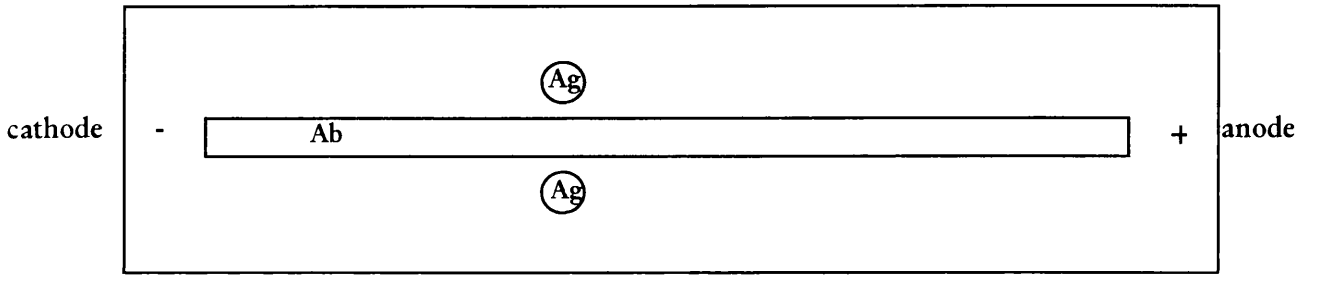


Figure 4.1 (c). Arrangement of wells used in single radial immunodiffusion assay. The gel already contains antibody and antigen samples are placed in the wells; samples of known antigen dilutions (dAg), used to produce a calibration curve from which it is possible to determine unknown antigen titre (uAg).

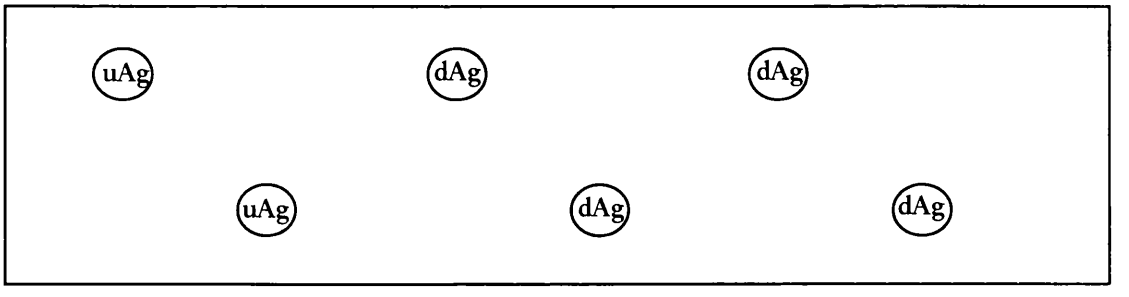


Figure 4.2 (a). Example of a double radial gel immunodiffusion showing reaction between the sheep anti-gull immunoglobulin IgY and various test antigens. Key: A - α-gull IgY (1st bleed); B - α-gull IgY (2nd bleed); C - gull yolk; D - gull sera; E - chicken yolk; F - chicken sera.

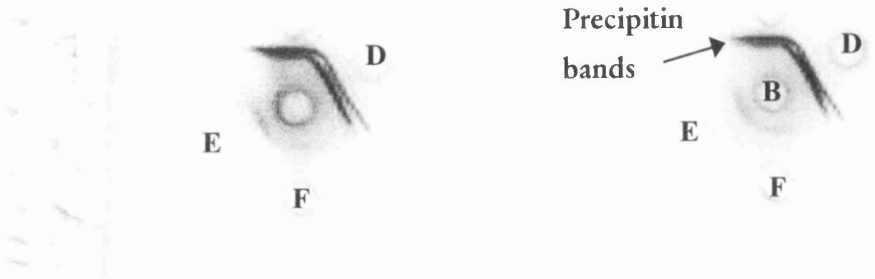


Figure 4.2 (b). Example of immunoelectrophoresis gel showing reaction of sheep anti-gull immunoglobulin IgY (in trough, refer to Figure 1b), with gull serum and yolk. Arrows indicate precipitin bands.

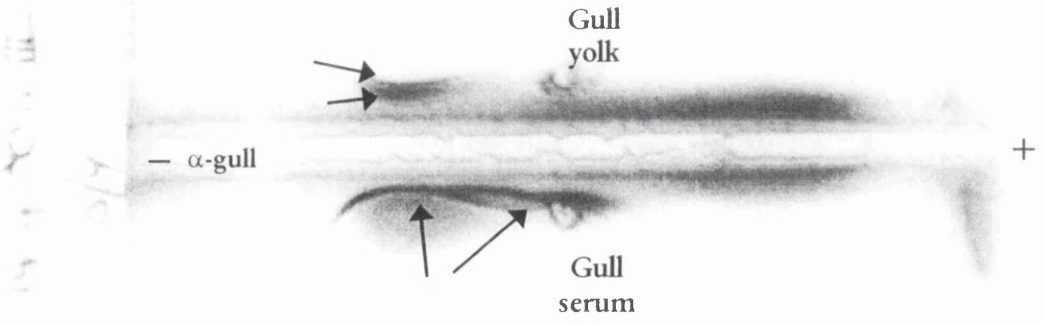


Figure 4.2 (c). Example of a single radial gel immunodiffusion, stained to show the reaction of sheep anti-gull immunoglobulin IgY incorporated into the gel, with increasing dilutions of gull serum (dS1-4) and test yolk samples (T1-2).

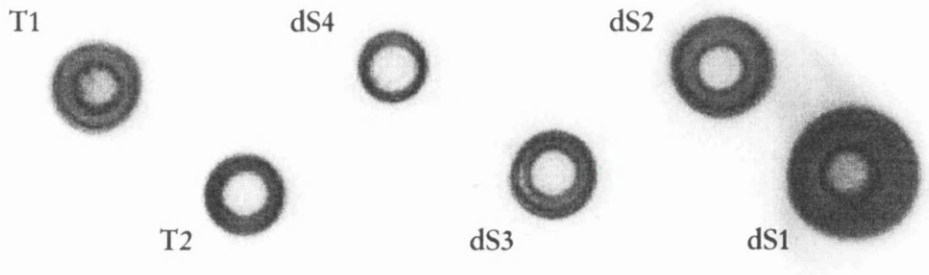


Figure 4.3. Calibration curve for single radial immunodiffusion

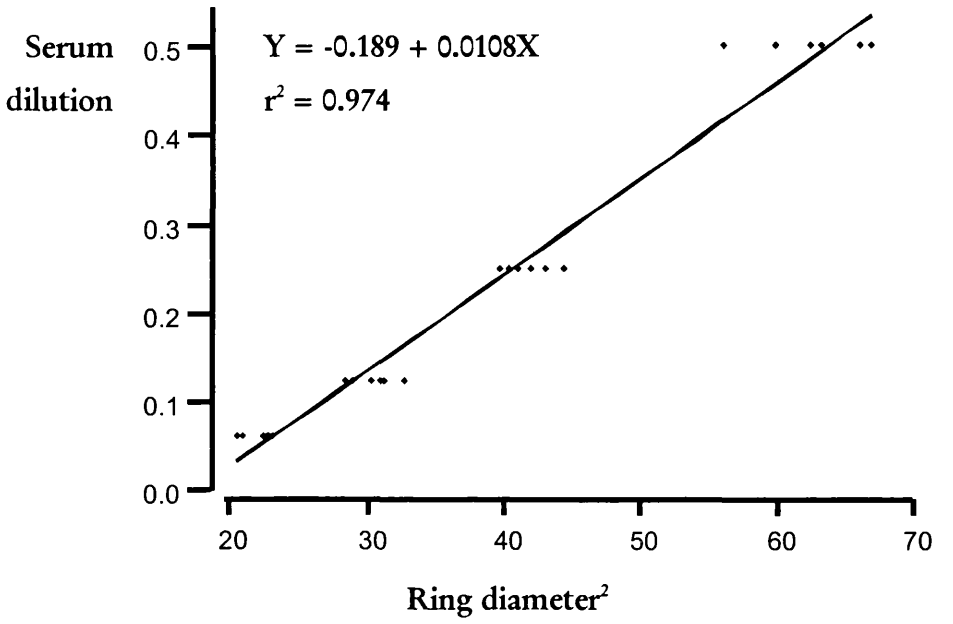
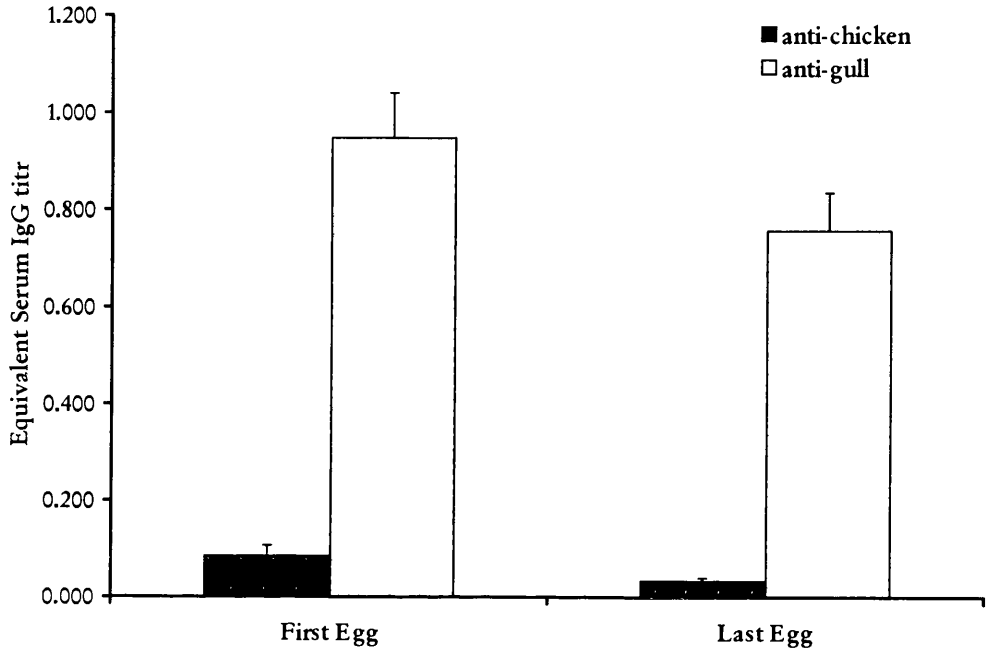


Figure 4.4 Comparison of yolk immunoglobulin titres measured using an ELISA with either anti-chicken (n = 9) or anti-gull (n = 22) reagents. Values are expressed in relation to immunoglobulin titres of homologous serum, used as the standard in each instance. Bars represent standard error.



CHAPTER 5[1] Egg quality changes with increasing egg production effort: a study of egg composition in extended clutches of lesser black-backed gulls, *Larus fuscus*

INTRODUCTION

Representing a considerable investment of her resources, a female bird deposits all the chemical nutrients required for the growth of an avian embryo within a sealed unit over a short period of time. Variation in both the total amount of resources allocated to a clutch of eggs and the distribution of those resources within a clutch can have a profound influence on both her offspring's and her own fitness (Bernardo, 1996). There is good evidence in lesser black-backed gulls (*Larus fuscus*) of a trade-off between the number and the quality of eggs (Nager et al., 2000). By removing eggs as they were laid, the number of eggs produced by lesser black-backed gulls was experimentally increased beyond the normal clutch size of three, at the expense of chick viability. Neither hatching nor fledging success of the eggs was related to their fresh mass, but was related to their position in the laying sequence. This suggests that changes in egg composition had a substantial effect on offspring survival. The objective of this study was to examine the chemical composition of experimentally induced extended clutches in more detail, in order to elucidate which aspects of the eggs are involved in this trade-off.

METHODS

This study was conducted in 1998 and 1999 at South Walney Nature Reserve, Walney Island, UK. The reserve supports a large mixed colony of gulls, with ~24 000 pairs of lesser black-backed gulls. Larus gulls usually lay a clutch of three eggs, but the laying of additional eggs can be experimentally induced by removal of the first and subsequent eggs within 12 hours of laying (Parsons, 1976; Monaghan et al., 1995); experimental birds then lay extended clutches. Eggs are normally laid at 2-day intervals and any eggs laid were removed at each nest until that bird stopped laying. Within a few hours of laying, each egg

[1] This chapter takes the form of an extended abstract, first published as Fidgett, A.L., Harper, E.J., Houston, D.C., Nager, R.G., and Surai, P.F., Avian egg quality changes with increasing egg production effort. In: Kirk Baer C.L., Editor. Proceedings of the 3rd Comparative Nutrition Society Symposium; 2000 Aug 4-9; Pacific Grove, California, USA.

removed was weighed to the nearest 0.1g, and maximal width and length were measured. Eggs were then separated into shell, yolk and albumen; wet weights of shell and yolk were recorded and used to calculate albumen weight. Samples of yolk and albumen were stored at -20°C prior to chemical analysis of composition. Analysis of egg composition was performed for three eggs (first, third and last egg laid), from 12 extended clutches (all greater than three eggs). Analyses were performed at the Central Nutritional Laboratory (Pedigree Masterfoods) and composition factors measured were water, lipid, crude protein, ash, selected essential amino and fatty acids by the methods outlined in Chapter 3 and Appendices I-IV. Levels of total carotenoids and fat-soluble vitamins A and E in the yolk were measured at the Scottish Agricultural College by methods described in Surai and Speake (1998). Relative yolk immunoglobulin titre was quantified using the enzyme-linked immunosorbent assay method developed for use with α-gull yolk immunoglobulin, as described in Chapter 4.

Results

On average birds were able to produce three times the normal clutch size ($\xi = 9.17 \pm 0.30$, range 5-13 eggs, n=12). Egg mass declined significantly over the laying sequence (Figure 5.1), but weight of the last laid egg (76.9 ± 1.44 g) did not fall below the weight of the last egg in a normal three-egg clutch (75.1 ± 0.82 g, n = 32; Nager et al., 2000). Due to the confounding effect of egg mass decline, results from all chemical analyses were expressed as concentrations and statistical tests were performed on arcsine transformed data. Eggs laid at the end of a sequence contain relatively less protein and lipid and relatively more water than earlier-laid eggs (Figure 5.2). On a finer scale, the weight of amino and fatty acids declined in absolute terms within an extended sequence, but relative to egg mass remained at the same concentration. This was not the case for vitamin E and total carotenoids. There was a highly significant decline in the levels of these compounds from first to third eggs and the decline appeared to plateau after the third egg, since there was no further significant decline between third and last laid eggs (Figure 5.3). Relative yolk immunoglobulin titre declined significantly within an extended sequence (Figure 5.4).

Discussion

Although fresh egg mass declined significantly with sequence position, increased egg production did not result in a reduction of egg mass below that of a normal third (and

hence last) laid egg. Smaller eggs contained most major nutrients in the same proportions as larger eggs, suggesting a blueprint for egg composition exists within the female, with limited scope for variation. That the last egg laid in extended clutches was not smaller than third eggs in normal clutches indicates the probability of a minimum size threshold below which an egg is unlikely to hatch and survive.

Earlier laid eggs contained significantly greater quantities of vitamin E and carotenoids, a phenomenon also observed in normal three-egg clutches (Royle et al., 1999). Both compounds are powerful antioxidants that protect both against peroxidative damage during development and the oxidative stress associated with hatching (Surai et al., 1996). Another property attributed to carotenoids is their ability to enhance the immune system and last-laid eggs have been demonstrated to contain significantly less immunoglobulin G (IgG) than earlier laid eggs. IgG, often referred to as IgY because it is deposited in yolk, is the avian equivalent of maternally-derived passive immunity. Thus the differential mortality of chicks hatching from eggs laid later in a sequence observed by Nager et al. (2000), may result from them having suffered more oxidative stress during development or having an increased susceptibility to pathogens.

While extended clutches are not a common natural occurrence, they demonstrate the extremes of egg production and how seriously an embryo may be compromised by very small changes in egg composition. However, it is possible to override this effect by means of supplementary feeding. Avian captive breeding programmes have utilised the practice of pulling eggs from a female to maximise reproductive effort in any one breeding season. Without due attention to the nutrition of the laying female this practise may in fact negate the benefits of increased egg yield by producing eggs of poorer quality which either fail to hatch, result in weakened offspring or have a biased sex ratio.

Figure 5.1 Mean (± SE) fresh mass (g) of eggs from experimentally extended clutches in relation to their position in the laying sequence. Egg mass declined with position in the laying sequence (repeated measures ANOVA: $F_{2,11} = 7.1040$; $p<0.01$).

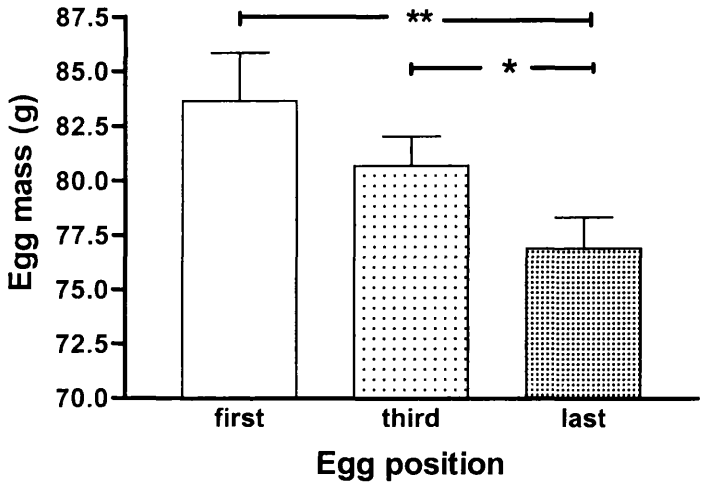
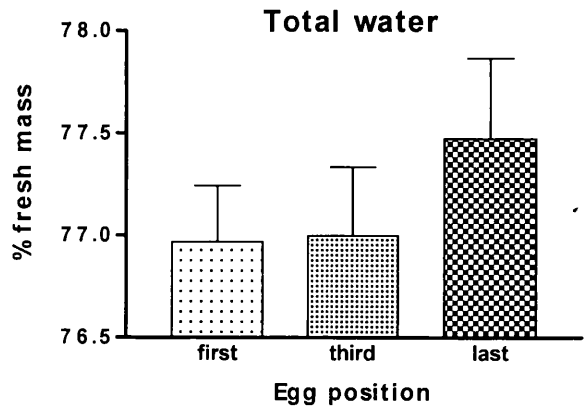


Figure 5.2 Mean (± SE) proportion of a) water, b) lipid, and c) protein in *L. fuscus* eggs, from experimentally extended clutches in relation to their position in the laying sequence.

a)



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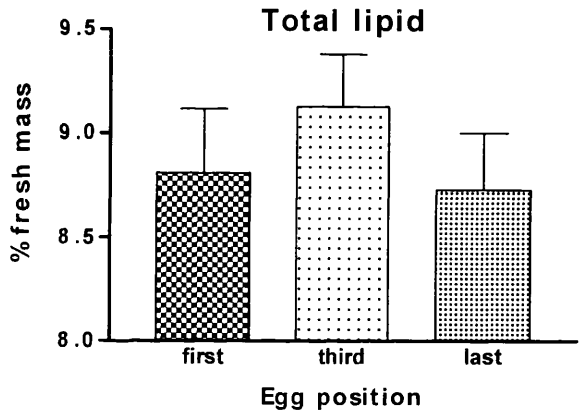


c)

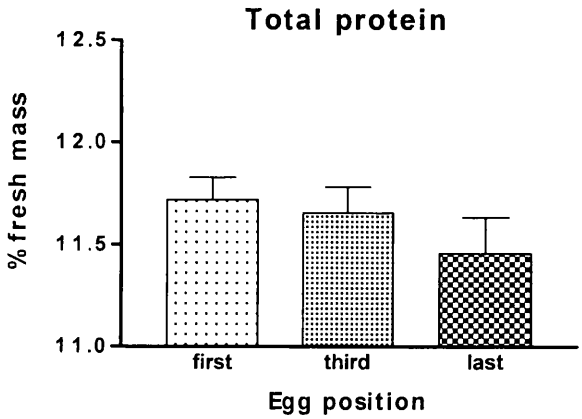
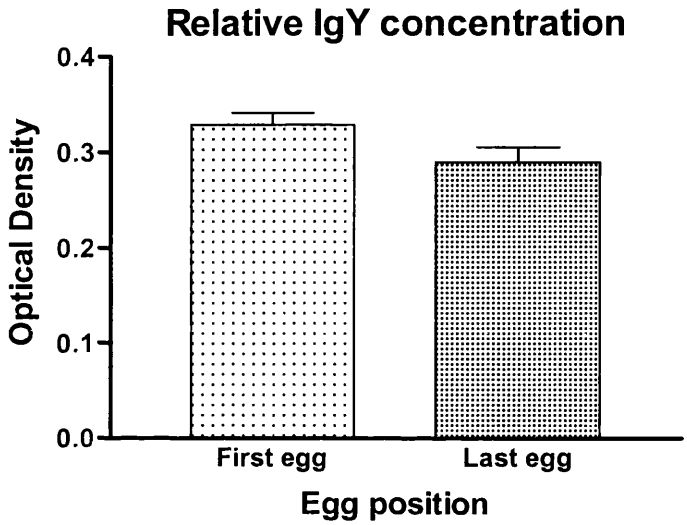


Figure 5.3 Mean (± SE) concentration (μg/g fresh yolk mass) of vitamin E (●) and total carotenoids (■) in eggs from experimentally extended clutches in relation to their position in the laying sequence. Concentrations declined with sequence position (repeated measures ANOVA: vitamin E - $F_{2,13} = 31.91$; $p<0.001$; carotenoids - $F_{2,13} = 26.95$; $p<0.001$), with the third egg and last eggs containing significantly less vitamin E and carotenoids than first eggs.

Figure 5.4 Mean (± SE) relative immunoglobulin (IgY) concentration from experimentally extended clutches of *L. fuscus* eggs. Concentrations declined with sequence position (paired t-test, t = 2.63; d.f. = 21; $p < 0.05$), with last-laid eggs containing significantly less immunoglobulin than first-laid eggs, after correcting for differences in egg mass



CHAPTER 6 Changes in lysozyme activity in albumen of lesser black-backed gulls (*Larus fuscus*) eggs, in relation to position in the laying sequence.

INTRODUCTION

The egg, primarily a store of food for the avian embryo, sometimes becomes the passive carrier of micro-organisms, which may avail themselves of its nutrients. The interior of the new-laid egg is usually free of micro-organisms, largely because of the natural protection provided by the egg's physical structure and by the chemical composition of the albumen. However, contamination of the egg contents occasionally occurs, either before the egg is laid or shortly thereafter, and as a result the egg may eventually decompose. Much of the published work on the natural mechanisms present in birds eggs to prevent microbial damage has been conducted with hens' eggs in order to improve their shelf-life.

Modes of contamination

The surface of the eggshell is usually covered with many species of bacteria and fungi, yet internal contamination remains low. There are two potential explanations for internal contamination: bacteria may be incorporated into the egg during formation, either in the ovary or oviduct; or micro-organisms may penetrate the eggshell after the egg is laid. Romanoff and Romanoff (1949), labelled these as congenital or extragenital modes of contamination respectively. Salmonella bacteria present in yolk are a classic example of congenital contamination. The bird is infected via the digestive tract from where bacteria pass into the blood stream and are transported to the ovary, at which point the bacteria may be deposited in yolk (Romanoff and Romanoff, 1949). More recent investigations suggest that Salmonella infection is in fact more commonly derived from oviducal infection than ovarian infection (Humphrey, 1994). Since the oviduct is an organ that opens into the body cavity at one end and the cloaca at the other, it offers double the source of infectious exposure. While it is generally supposed that foreign organisms are removed from the oviduct by a variety of mechanisms including peristaltic action directed towards the cloaca, records exist of foreign objects such as parasitic worms, insects, pebbles and feathers being found inside eggs. This clearly indicates that the potential for contamination via the cloaca cannot be overruled (Romanoff and Romanoff, 1949).

After laying, the eggshell provides a physical barrier against invasion of bacteria, but it is not completely impervious due to the structural pores necessary for respiration and water balance. That fungal mould could easily penetrate the eggshell via these pores was demonstrated as early as 1851 by von Wittich. Rapid changes in environmental temperature also favour microfloral invasion since they often result in condensation. If the external temperature is lower, egg contents contract and draw water (and any surface bacteria) through the shell by suction. Another mode of extragenital contamination for newly laid eggs may be to draw in bacteria with the air that forms the air cell (Romanoff and Romanoff, 1949).

Contamination can occur in both freshly laid eggs and those stored for a period of time, but to survive and flourish within the egg, the micro-organisms really need to reach the yolk. Both the physical organisation of the egg contents and their chemical composition together form an efficient defensive system for impeding the progress of micro-organisms.

Physical defence mechanisms

Physical defences or barriers comprise the shell, the shell membranes and finally the albumen. Thick versus thin albumen and arrangement of albumen proteins provide an effective obstacle impeding access to the yolk. If the yolk has been broken and mixed with the albumen however, bacteria will grow luxuriantly (Romanoff and Romanoff, 1949; Board et al., 1994).

Board et al., (1994) emphasises the compartmentalised nature of the egg. Within 'table' eggs (i.e. ones produced for human consumption and therefore unfertilised) these 'compartments' retain their integrity through protracted storage. But their spatial arrangement and physical characteristics do change appreciably, largely due to the decay of the albuminous sac and changes in the chalazae (Figure 6.1), which result in the yolk moving from a central to a peripheral location within the egg. Change in yolk location is also due to diffusive loss of water from the albumen to the atmosphere around the egg (Ar, 1991), as well as the migration of water from the albumen to the yolk (Burley and Vadehra, 1989). Albumen liquefaction probably serves to liberate macromolecules, glucose, and essential ions and to facilitate their movement to the blastoderm (Brake et al., 1996). It may also serve to reduce the barrier to gaseous diffusion imposed by the albumen (Meuer and Baumann, 1988). The albumen exhibits the most obvious and thoroughly documented

postovipositional changes of any egg component and is strategically positioned between, and has been reported to interact with, the shell membranes and the perivitelline layer (layer surrounding the yolk) (Brake et al., 1996). The result of these changes are that over time the yolk moves closer to the shell membrane and becomes more vulnerable to microbial invasion.

Chemical defence mechanisms

The hostile nature of albumen with respect to micro-organisms such as typhoid bacilli, was noted as early as 1890 by Wurtz. Laschtschenko (1909) found that albumen left in an open, sterile dish remained free of bacterial growth for as long as 2 months. Stuart & McNally (1943) very elegantly demonstrated that chemical defences were contained in the albumen, by observing the extent of contamination in shell membrane, albumen and yolk during 80 hours incubation of eggs smeared externally with a culture of *Pseudomonas aeruginosa*. After rapidly penetrating through the shells, bacteria multiplied in the albumen for a short time and then rapidly decreased in number. Contamination of the yolk was delayed and relatively light (Figure 6.2). The hostile nature of albumen may be due in part to the inability of bacteria to utilise native proteins (e.g. ovalbumin). Bacteria also require specific minerals e.g. calcium and iron, which are not bioavailable within albumen, and high alkalinity may also be a factor (Board et al., 1986).

As described in previous chapters, albumen is predominantly composed of water (85-91% from the range of eggs sampled in Chapter 3). Protein is the main component of albumen dry matter and Table 6.1 lists the major proteins in hens' egg albumen, together with their relative proportion and characteristics. Although certain properties are ascribed to each of them, the physiological roles for many of these proteins are not yet well understood. Ovalbumin is the most abundant. While its structure and composition have been extensively described, its function remains unclear other than having a amino acid composition similar to a family of protease inhibitors. It may also serve as a source of amino acids for the embryo when it is nearly full-grown. Ovotransferrin, formerly known as conalbumin, is one of a group of closely-related proteins - the transferrins, which are also found in blood, liver and egg yolk. These proteins all share a binding affinity for iron and certain other metals, and are largely responsible for the transport of iron in higher animals. In the egg ovotransferrin does not contain iron, so possibly one of its functions is to maintain a low concentration of free iron and thus deny this essential mineral to micro-

organisms (Burley and Vadehra, 1989). Ovomucoid is a glycoprotein and is the main inhibitor of proteolytic enzymes in avian eggs. One hypothesis for the function of protease inhibitors in eggs is to control the breakdown of proteins into their constituent amino acids during embryogenesis (Burley and Vadehra, 1989). Ovomucin, previously referred to as egg mucin, is also a glycoprotein and although it occurs as a relatively small proportion of the albumen, it is an extremely important component of 'table' eggs. This is because ovomucin is responsible for the gel structure of the 'thick' albumen and therefore the albumen as a whole. It may function as a physical means of defence, impeding the movement of invading micro-organisms, however it may also have antiviral properties (MacDonnell et al., 1953). The lysozyme of avian egg albumen (and also present in the shell and vitelline membrane), belongs to a class of enzyme that lyses the cell walls of gram-positive bacteria (Burley and Vadehra, 1989). It is this direct capacity to destroy bacteria that has led to the assumption that lysozyme is the predominant chemical defence mechanism in albumen.

Lysozyme

The failure of bacteria to grow in albumen is chiefly attributed to substances that destroy bacteria. Of these, lysozyme is the best known, first described by Alexander Fleming in 1922 as a 'remarkable bacteriolytic element found in tissues and secretions'. Fleming named the substance and it marks his first significant step in the search for antibacterial substances which culminated later in his discovery of penicillin. In his search to determine the distribution of this substance he noted that lysozyme was not only found in albumen, but is also present in tears and many other secretions obtained from plant and animal tissues, from a great range of taxonomic groups (Fleming, 1922).

Although Fleming's description of lysozyme went on to be eclipsed by his discovery of penicillin, biochemists were intrigued to determine the mechanism of enzyme action and lysozyme provided an excellent means of investigation (Chipman and Sharon 1969). Lysozyme is easily isolated and purified from hen's egg albumen (often referred to as hens egg white or HEW in older papers). The egg white of the chicken and other galliform birds contains lysozyme to the extent of 2-4 % of the total egg white protein, a remarkably high concentration for a single enzyme. It is stable and has a fairly low molecular weight and as such has become one of the most thoroughly investigated proteins. For some time it was of interest to researchers investigating bacterial cell walls and much of the work used

Micrococcus lysodeikticus (a very sensitive bacterial strain), as a substrate for lysozyme activity. Lysozyme hydrolyses the beta linkage between residues of N-acetylmuramic acid and N-acetylglucosamine, and this discovery led to the elucidation of bacterial cell wall structure (Salton, 1957).

Lysozyme actually refers to a group of related proteins. Since birds' egg albumen is such a rich source of the protein, many species other than domestic chickens have been studied, principally to investigate the structural, chemical and crystallographic variations of the molecule. The list of bird species includes: ostrich (Schoentgen et al., 1982); cassowary (Thammasiriak et al., 2001); various Phasianidae (Araki et al., 1990, 1991, 1994, 1998a); goose (Simpson and Morgan, 1983); swan (Morgan and Arnheim, 1974) and pigeon (Acharya et al., 1994). The complete amino acid sequence of a lysozyme collected from reptile eggs has also been described (Araki et al., 1998b).

Lysozyme is characteristically identified by its clarification of a suspension of cells of the bacterium *Micrococcus lysodeikticus*, a technique used since Fleming's discovery of the enzyme (Fleming, 1922). One method used to detect lytic activity is the lysoplate technique, whereby samples of unknown lysozyme activity are placed in wells punched in petri dish with an agar layer containing *M. lysodeikticus*. After overnight incubation the resultant zones of lysis, denoted by clear area of agar around the wells, can be compared to those formed by hen egg-white lysozyme standards to estimate the lysozyme content of the unknown sample (Birkbeck, 1982). This method has been used to measure and determine lysozyme activity in marine bivalves (McHenery et al., 1979) but Fleming and Allison (1924) also used this method to compare the lytic ability of albumen from several bird species. They noted varying degrees of bacteriolytic activity; hen's albumen being more active than that of the moorhen *Gallinula chloropus*, thrush *Turdus philomelos* or wagtail *Motacilla alba* (Figure 6.3).

Lysozyme activity may also be measured by the reduction in optical density of a suspension of *M. lysodeikticus* over a fixed period of time. A reduction in optical density suggests that *M. lysodeiktus* cells are being lysed and the contents are leaking out. Commercial, crystallised hens' egg-white lysozyme is again used as a reference standard (Wardlaw and McHenery, 1982). Using this method Feeney et al., (1966) reported low levels of lysozyme activity in egg white from the Adelie penguin *Pygoscelis adeliae*, at approximately one-

hundredth of the activity of lysozyme in chicken egg white. An even lower level of lysozyme was found in egg white from the Royal penguin *Eudyptes schlegeli* (Feeney and Allison, 1969). The low level of lysozyme in egg white from the Adelie penguin was suggested to be the result of the less intense selection pressures for antibacterial substances in the egg white of a bird which nests in a comparatively cleaner and colder place, such as the Antarctic. However, egg albumen from the fairy penguin *Eudyptula minor*, a species inhabiting a more temperate climate, was also found to contain low levels of lysozyme (Manwell and Baker, 1973).

Aims of this chapter

Both the crystallographic and chemical structure of lysozyme have been documented for many bird species other than domesticated birds, yet there has been comparatively little work done on levels of lysozyme activity in wild bird eggs and their ecological importance. To the best of my knowledge, the potential for within-species variation between eggs does not appear to have been investigated for any wild bird species. I chose to examine this phenomenon in eggs collected from the lesser black-backed gull (*Larus fuscus*). There were two main aims of this study. Firstly I wanted to investigate whether there was variation in lysozyme activity in eggs, both between eggs laid within single clutches, and between females. The eggs used were collected from gulls which had been manipulated to lay experimentally extended clutches, the hypothesis being that eggs from these extended clutches would demonstrate the extremes of egg production; within a clutch for a single female, and between females. As such, differences in egg composition might be more pronounced. It is known that hatchability of those extended clutches declines from 85% to 30% from first to last laid eggs (Nager et al., 2000), and reduced lysozyme action may contribute to this decline in hatchability success. Secondly, I considered how lysozyme activity levels in this species of wild bird compared with known values for poultry, and speculate what might be the reasons for any disparity.

MATERIALS AND METHODS

Gull egg albumen samples

This study was conducted in 1999 at South Walney Nature Reserve, Walney Island, UK. The reserve supports a large mixed colony of gulls, with ~24 000 pairs of lesser black-backed gulls *Larus fuscus*. As described previously in Chapters 4 and 5, *Larus* gulls usually lay a clutch of three eggs, but the laying of additional eggs can be experimentally induced

by removal of the first and subsequent eggs within 12 hours of laying (Parsons, 1976; Monaghan et al., 1995); experimental birds then lay extended clutches. Eggs are normally laid at 2-day intervals and any eggs laid were removed at each nest until that bird stopped laying. Within a few hours of laying, each egg removed was weighed to the nearest 0.1g, and maximal width and length were measured. Eggs were then separated into shell, yolk and albumen; wet weights of shell and yolk were recorded and used to calculate albumen weight. Samples of yolk and albumen were stored at -20°C prior to various chemical analysis described in other chapters. Analysis of lysozyme activity was performed for all eggs in 18 extended (all greater than three eggs) clutches (n = 121 eggs).

Lysozyme activity assay

As mentioned earlier, lysozyme can be characteristically identified by its clarification of a suspension of cells of the bacterium *M. lysodeikticus*. The decrease in light scattering registers as a decrease in absorbance by a spectrophotometer and is quantified as a decrease in optical density, usually over a fixed period of time. Hens' egg white lysozyme was used as the standard and dilutions of the standard and test samples were loaded (in triplicate) onto a 96-well microtitre plate. The bacterial suspension of *M. lysodeikticus* was only added at the plate reader, the microtitre plate was then immediately loaded into the reader and the reader software activated to run a kinetic assay. The kinetic assay was programmed to read the optical density of each well on the plate at 5 minute intervals for 40 minutes, at a wavelength of 540nm. It was then possible to plot a graph of change in OD per minute against the lysozyme standards, in order to determine the lysozyme concentration of the unknown samples.

Preparing standards

Hens' egg white lysozyme, available from Sigma (St. Louis, USA), was used as the standard. A stock solution of 40 μg/ml hen egg white lysozyme in phosphate buffered saline was prepared, and then a series of doubling dilutions in buffer was made from this stock. Each dilution was vigorously mixed before carrying forward to the next dilution. A zero or blank, of buffer alone, was also prepared.

Preparing Micrococcus lysodeiktus substrate solution

M. lysodeikticus (Sigma) was suspended in buffer at a concentration to provide an initial OD of 0.4 since this puts the assay in range for most accurate OD measurements (Helen Reid,

pers. comm.). A solution of 0.4 mg *M. lysodeiktus* in 1ml of buffer provided the optimal OD of 0.4 and *M. lysodeiktus* resuspended easily and evenly in buffer.

Setting up the microtitre plate

A 96-well microtitre plate was used, allowing a series of blanks, standards and test samples to be assayed in triplicate according to the template in Figure 6.4. 20 μl of each standard and each test sample was aliquoted into the wells. One triplicate set of wells contained only buffer alone (200 μl), to give a background (or blank) reading. Preliminary trials found it difficult to replicate results between wells with undiluted gull albumen samples, perhaps due to the viscosity of the sample material. Therefore all gull egg samples were diluted 1:1 in buffer and mixed thoroughly. Another factor that seemed to have an effect on repeatability of the assay was air bubbles formed while pipetting the gull albumen samples into the wells, again probably due to the sample viscosity. Therefore, before the bacterial solution was added all air bubbles were eliminated from the wells using a fine gauge hypodermic needle, to ensure adequate mixing of each test sample and the *M. lysodeiktus* solution.

Immediately before starting to the kinetic assay, 180 μl of the *M. lysodeiktus* solution was pipetted into each well using a multi-pipette, with care taken to ensure that the same volume was drawn into each tip at all times. Since any lysozyme present will start to act on the bacterial solution immediately and accurately timed measurements are required in order to measure concentration, this last stage was done at the MRX Microplate Reader (Dynatech Laboratories, Virginia).

Running a kinetic assay

Biolinx software (Version 2.20, Dynatech Laboratories, Virginia), was used to run a kinetic assay, so called because it reads the optical density of each well on the plate (at a specified wavelength), at regular intervals over a pre-set period. The settings I used were 5 minute intervals for a period of 40 minutes, at a wavelength of 540nm. Since gradual settling of the cell suspension can cause an apparent decrease in light scattering, giving spurious results at low lysozyme levels, the software was set to shake the plate for 10 seconds before recording optical density at each pre-set interval. Experience of using this assay to measure low levels of lysozyme activity in shellfish suggested that the greatest changes in optical density were likely to occur over the first 10 minutes (H. Reid, pers. comm.). Optical

density/min values for this period were much higher than over the entire 40 minute assay. However, as my assay was also being used to measure unknown, but potentially very small amounts of lysozyme, I felt that running the assay for a longer time would ensure low levels of the enzyme could be detected.

The Biolinx software used the values obtained from the hen's egg white standards to produce a graph of optical density/minute against known concentrations for each plate. None of the calibration curves had an $r^2 < 0.98$, and a further check of the plate's integrity was whether or not the optical density of the buffer-only wells had remained constant over the entire assay period of 40 minutes. The optical density for each test sample (based on the mean of the replicates) was compared to the calibration curve and the software automatically calculated the lysozyme activity in μg/ml, taking into account the 1:1 dilution factor of the test albumen samples.

RESULTS

On average birds were able to produce three times the normal clutch size of 3 eggs (ξ = 9.17 ± 0.30, range 5-13 eggs, n= 18. Egg mass declined significantly over the laying sequence (Figure 6.5), but weight of the last laid egg did not fall below the weight of the last egg in a normal three-egg clutch, as shown by Nager et al., (2000). Due to the confounding effect of egg mass decline, results from all chemical analyses were expressed as concentrations and statistical tests were performed on arcsine transformed data.

Lysozyme concentration in all the eggs sampled ranged from 1.8-32.4 μg/ml (n = 121; Figure 6.6). Since clutches varied in length, changes in clutch lysozyme concentration were represented by the slope calculated from regressing concentration against egg position in the laying sequence (Table 6.2). Using all the slopes from the regression of egg position against lysozyme concentration, the result was highly significantly different from 0 (one-sample t-test, T =-3.13, p = 0.0061, n =18; Table 6.2), indicating that lysozyme concentration declined with egg position over the laying sequence. Furthermore, last laid eggs had lower lysozyme levels than the first eggs in a sequence, although this difference was not significant (repeated measures ANOVA, $F_{2,16}$ = 1.24, p = 0.298; Figure 6.7). The pattern of decline in lysozyme concentration with laying sequence was also evident for the

3rd egg in extended sequences, which would typically be the last laid egg of a 'normal' (unmanipulated) clutch (Figure 6.7).

The mean weight of first laid eggs was 79.7 g (ranging from 70.5-90.4 g) and the mean proportion of albumen contained within was 66.1% (range 61.4-72.7 %; Table 6.2). There was a concomitant decline in absolute mass of the eggs as the egg number increases (Figure 6.5), thus earlier eggs, which were larger, contained proportionally more albumen and lysozyme. There was no relationship between this decline in lysozyme in later-laid eggs and the final clutch size, start of laying date or mass of the first-laid egg, all well-recognised indicators of female quality. Nor was the decline related to the absolute albumen mass of first-laid eggs, or the proportion of albumen they contained (Table 6.3).

From Figure 6.6 it is apparent that there was considerable variation in the lysozyme concentration of first-laid eggs. However, no relationship was found between the final clutch size, start of laying date, mass of the first egg or its albumen content, either absolute or as a proportion of total mass (Table 6.3).

DISCUSSION

There was considerable inter-female variability in the lysozyme content of first-laid eggs, which did not appear to correlate with any standard measures of female quality (clutch size, start of laying date, mass of first-laid egg; Table 6.3). However, within a clutch, later-laid eggs exhibited demonstrably less lysozyme activity and therefore had a lower lysozyme concentration. Since egg mass also declined significantly with laying position, these later-laid eggs were also more likely to contain less lysozyme in absolute terms.

Variation in avian egg albumen is widely recognised (Brake et al., 1997). Quality of albumen declines with storage (Hurnik et al., 1978) and hen age (Burley and Vadehra, 1989), while albumen protein content has also been reported to decline with hen age (Cunningham et al., 1960). Although much of what is known about changes in albumen quality has been determined from research conducted on domestic laying chickens to improve egg shelf-life, perhaps some of the changes in egg composition relating to increased effort or age may be relevant to older, or artificially more productive wild birds.

Lysozyme activity levels in *Larus fuscus* were much lower than for hens' eggs tested using the same method, having approximately 1/100 of the lysozyme concentration (ξ 16 $\pm$ 5 μg/ml for gull eggs, versus ~1500 μg/ml found in hens' eggs). These values may be underestimated because, when egg albumen is more concentrated, the activity of lysozyme is decreased as a result of an association between lysozyme and other substances (Romeo and de Bernard, 1966). Lysozyme activity in the albumen of a penguin species was observed to be lower again; the relative activity of fairy penguin *Eudyptula minor* albumen to chicken albumen measured from 1/250 to 1/1070 depending on the dilution of material being tested (Manwell and Baker, 1973). The possibility that there is a positive correlation between the levels of lysozyme in egg white and dirtiness of nesting habits in birds is an attractive hypothesis. If such a correlation were to be strong, then one would expect to find high levels of lysozyme activity in the egg white of penguin species which nested in warmer climates where a greater diversity of bacterial species were present. Presence of low levels of lysozyme in egg albumen from three species of penguin (fairy penguin, Adélie penguin *Pygoscelis adeliae* and Royal penguin *Eudyptes schlegi*), suggests this may simply be a general characteristic of the Sphenisciformes (Feeney et al., 1966; Feeney and Allison, 1969). Certainly a species nesting in warm temperate regions (the fairy penguin) had no more lysozyme in its egg albumen than species nesting near the Antarctic ice (the Adélie and Royal penguins; Feeney et al., 1966; Feeney and Allison, 1969; Manwell and Baker, 1973).

However, there are very few other data for comparison. Feeney and Allison, (1966) mention low (undefined) levels of lysozyme measured in banded plover (*Zonifer tricolor*), intermediate levels for the masked lovebird (*Agapornis personata*). Manwell and Baker (1973) refer to lysozyme activity approximately 1/40 that of chicken, determined from the egg albumen of crested tern (*Sterna bergii*). Hens may have incidentally had more lysozyme in their eggs and this trait has been selected for and enhanced. The phenomenon of hen's eggs having greater concentration of lysozyme than other bird species was recorded as early as 1924 (Fleming and Allison, 1924; Figure 6.3), before the intensive farming boom of the fifties. Therefore particularly high lysozyme in hens' eggs is unlikely to be a direct result of intensive farming, but could have been selected for since then.

It has been suggested that one reason for the evolution of complex antimicrobial defence systems in eggs is because asepsis during egg formation cannot be assured and breeding

success depends on this defence (Tranter and Board, 1982). In nature the chick is first exposed to infection at pipping and in the wild this means exposure to microflora that has built up in the nest during incubation. Furthermore it's important to recognise that in nature the non-specific defence system of a fertilised egg would need to be effective for a short period only – the interval between the laying of the first egg and the onset of incubation (as compared with 'table' eggs). Within a short while of incubation, new compartments have formed and certain resources essential to embryogenesis redistributed in a fertile egg. Thus within 9 days the yolk is enveloped by the cellular yolk sac membrane (Burley and Vadehra, 1989) and as incubation progresses a large amount of the water reserve in albumen has been transferred to a part of the yolk, dramatically changing the environment within which lysozyme functions (Deeming, 1991).

So, despite extensive structural, chemical, crystallographic, genetic, immunological and evolutionary studies devoted to lysozyme, its biological role generally is still not exactly known, much less its specific function within avian egg albumen. The albumen manages to be hostile to bacterial and fungal cells but not those of yolk or chorioallantoic membranes. Lysozyme contributes to the antimicrobial defence of avian eggs and this defence mechanism is unique in that it operates in unincubated eggs, and in the absence of host cells or a vascular system. But what is lysozyme's biological role, since there do not appear to be many bacteria pathogenic to vertebrates which are susceptible to lysis by lysozyme? Nearly all bacteria have a layer in their cell walls that can be digested by lysozyme (Salton, 1964) and Chipman and Sharon (1969) speculate that its role is to digest cell wall debris from bacteria killed in other ways. That idea has been supported by recent research suggesting a close functional relationship between lysozyme and immunoglobulins related mainly, but not exclusively, to bacteriolysis. Lysozyme and secretory immunoglobulins (mainly IgA) show a similar distribution in various body fluids (Jollès and Jollès, 1984).

On its own, the decreasing lysozyme concentration observed in experimentally extended gull egg clutches may not be biologically significant but, combined with declines in other components of the egg as described in Chapter 5, it may contribute cumulatively to the decreased hatchability and fledging success observed by Nager et al., (2000). Natural variation in 3-egg clutches needs to be observed, and comparisons of lysozyme activity in a broader range of wild bird species might begin to throw light on why wild birds have so much less lysozyme than domesticated species.

Table 6.1 Properties of the main proteins of hen egg albumen (compiled from Stevens, 1991; Burley and Vadehra, 1989; Board et al., 1994)

Protein	Amount in albumen (%)	Characteristics
Ovalbumin	54	Possible enzyme inhibitor and/or metal chelation
Ovotransferrin	12	Chelation of metal irons, particularly iron
Ovomucoid	11	Inhibition of trypsin
Lysozyme	3.4	Enzyme activity. Interaction with ovomucin
Ovomucin	3.5	Mucilaginous virus inhibitor
Ovinhibitor	1.4	Inhibition of several proteases
Ovomacroglobulin	0.5	Protease inhibitor
Ovoglycoprotein	1.0	?
Ovoflavoprotein	0.8	Chelation of riboflavin
Avidin	0.05	Chelation of vitamin (biotin)

Table 6.2 Lysozyme concentration values for first laid egg (1st egg) in extended clutches. Clutch concentration is represented by the slope calculated from regressing concentration against egg position. Various other parameters of female quality are summarised for each clutch.

	Lysozyme Conc.			1st egg				
Nest ID No.	First Egg	Clutch (Slope)†	No. eggs laid	Date laid	April Day*	Total mass	Albumen mass	Albumen %
1	25.49	-1.1804	10	11-May-99	41	81.1	53.1	65.5
2	13.96	-0.3408	4	14-May-99	44	80.3	54.4	67.7
3	25.34	-0.4841	10	10-May-99	40	77.9	50.3	64.6
6	17.66	-0.2228	4	08-May-99	48	74.8	50.5	67.5
8	12.72	-0.103	8	14-May-99	44	79.3	52.6	66.3
13	10.69	-0.0313	11	06-May-99	36	82.6	54.5	66.0
14	19.76	-0.056	10	10-May-99	40	84.5	56.7	67.1
15	15.97	-0.5218	8	06-May-99	36	71.3	44.8	62.8
17	22.22	-0.6233	6	11-May-99	41	81.7	54.1	66.2
20	19.02	-0.0649	7	18-May-99	58	75.2	49.9	66.4
21	29.07	-1.2169	4	23-May-99	53	82.7	56.4	68.2
24[1]	16.97	-0.452	7	22-May-99	52	88.1	-	-
26	13.50	-0.3513	5	15-May-99	45	74.6	48.3	64.7
29	32.38	-2.7064	4	17-May-99	47	70.5	43.3	61.4
31	16.88	-0.0858	8	18-May-99	48	78.9	50.4	63.9
32[2]	25.32	-0.346	10	12-May-99	42	90.4	61.2	67.7
34	11.76	0.2485	6	10-May-99	40	85.4	62.1	72.7
35	11.37	-0.3011	4	18-May-99	48	75.1	48.7	64.8

Notes:

† Single value for clutch based on slope take from regression equation plotting lysozyme concentration against egg position in laying sequence.

*April day, number of days after April 1st.

[1]Yolk burst during preparation and calculation of albumen weight was not possible.

[2]No sample available for 1st laid egg, values presented are for 2nd egg in extended sequence

Table 6.3 Correlation values for lysozyme concentration of 1st egg, or decline in clutch (as represented by slope calculated from regression†), against various recognised measures of female quality, (n = 18). Since total egg mass, albumen mass and % albumen are not independent variables, their significance level is set at 0.05/n, where n equals the number of categories.

Measures of female quality	Lysozyme concentration 1st egg (r_s, P)		Lysozyme concentration Clutch (r_s, P)	
No. of eggs laid	0.01	0.98	0.30	0.23
April day*	0.18	0.48	-0.14	0.58
Total mass, 1st egg	-0.01	0.98	0.34	0.16
Albumen mass, 1st egg	-0.08	0.75	0.45	0.07
% Albumen, 1st egg	-0.26	0.31	0.52	0.03

Notes:

† Single value for clutch based on slope take from regression equation plotting lysozyme concentration against egg position in laying sequence.

*April day, number of days after April 1st.

Figure 6.1 The structure of a hen's egg a) at laying and b) after storage. From Board et al., (1994).

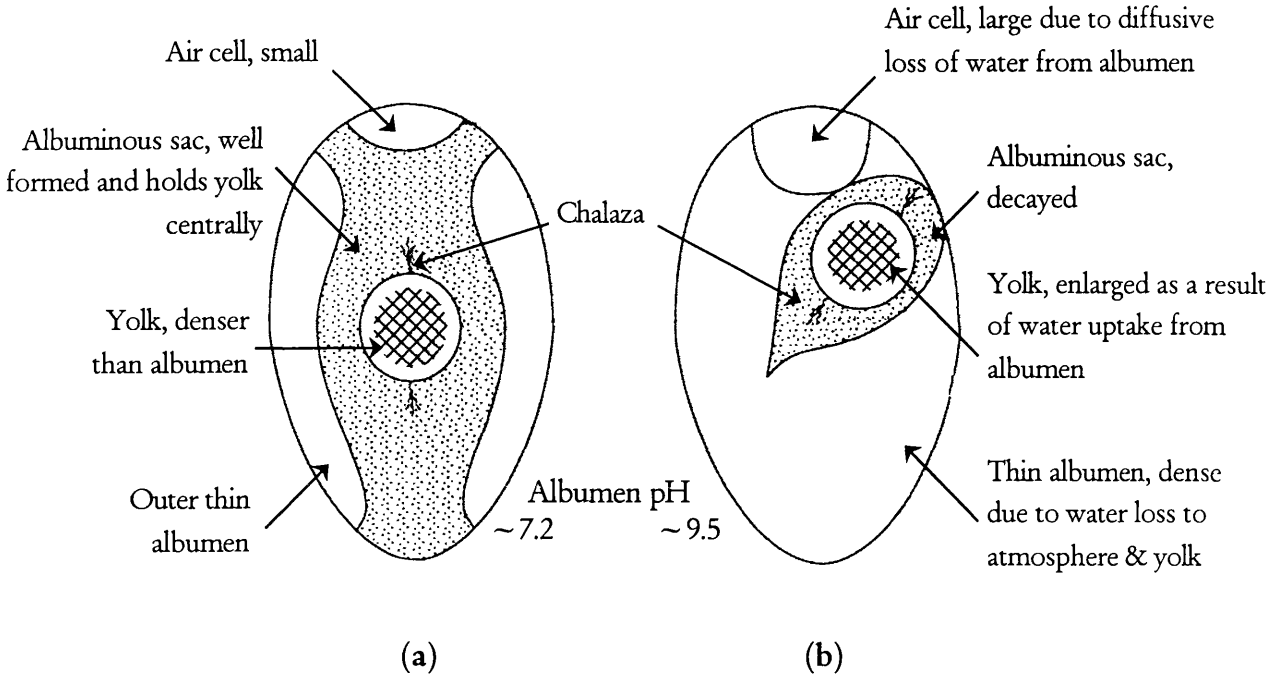


Figure 6.2 The extent of contamination in shell membrane, albumen and yolk observed during 80 hours of incubation of eggs smeared externally with a culture of *Pseudomonas aeruginosa* (after Stuart & McNally, 1943).

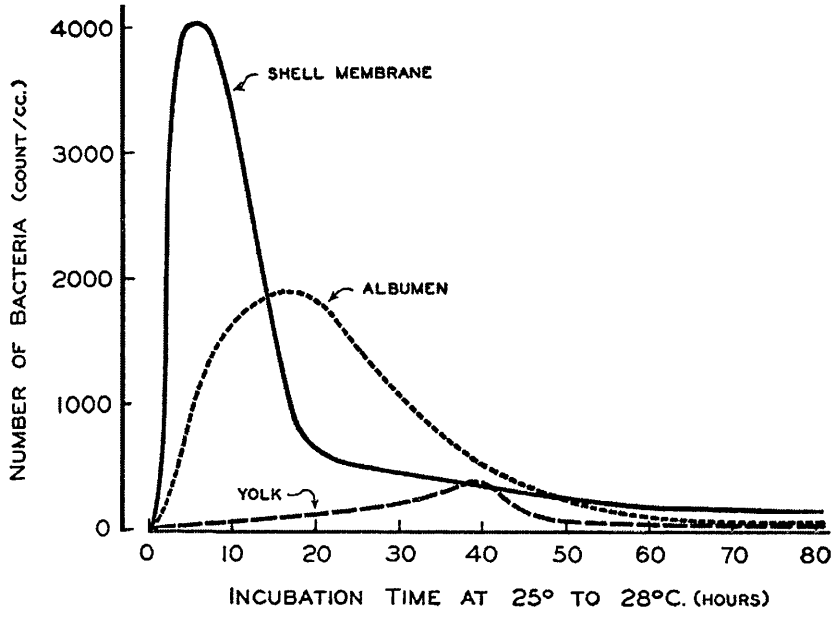


Figure 6.3 Comparison of the ability of albumen from the eggs of several species of birds to inhibit the growth of *Micrococcus lysodeikticus* on an agar plate (after Fleming and Allison, 1924).

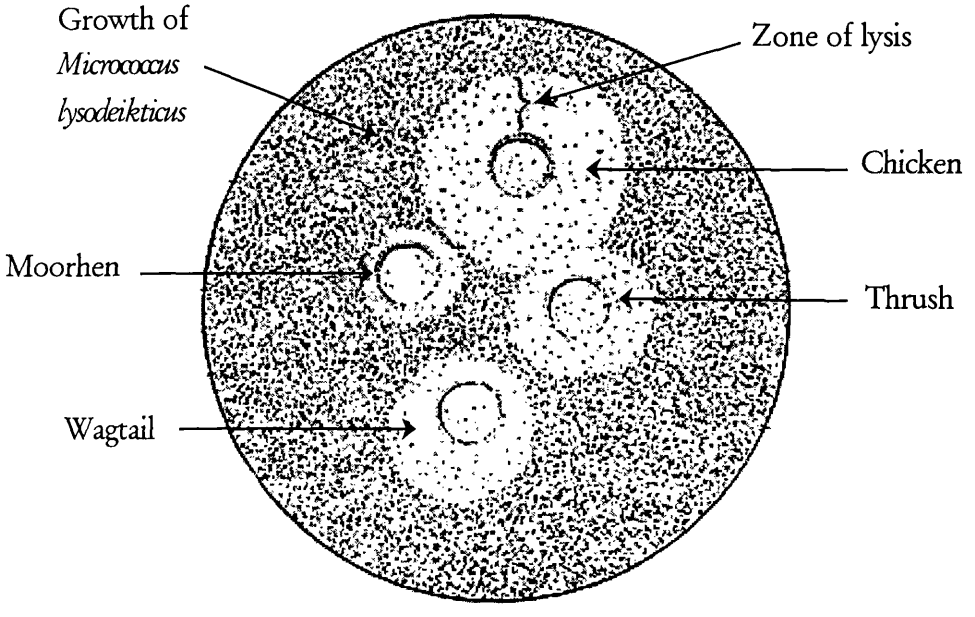


Figure 6.4 Template used for loading blanks (B), standards (S) & test samples (T) onto 96-well microtitre plate for lysozyme activity assay.

S1	S1	S1	T1	T1	T1	T9	T9	T9	T17	T17	T17
S2	S2	S2	T2	T2	T2	T10	T10	T10	T18	T18	T18
S3	S3	S3	T3	T3	T3	T11	T11	T11	T19	T19	T19
S4	S4	S4	T4	T4	T4	T12	T12	T12	T20	T20	T20
S5	S5	S5	T5	T5	T5	T13	T13	T13	T21	T21	T21
S6	S6	S6	T6	T6	T6	T14	T14	T14	T22	T22	T22
S7	S7	S7	T7	T7	T7	T15	T15	T15	T23	T23	T23
B	B	B	T8	T8	T8	T16	T16	T16	T24	T24	T24

Figure 6.5 Mean (± s.e.) fresh mass (g) of eggs from experimentally extended clutches in relation to their position in the laying sequence. Egg mass declined with position in the laying sequence (repeated measures ANOVA: $F_{2,11} = 7.1040$; $p<0.01$).

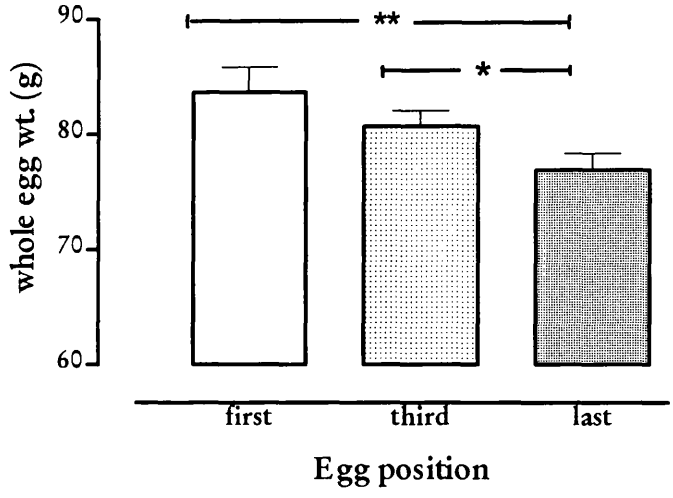


Figure 6.6 Scatter plot showing lysozyme concentration of all eggs sampled, against the order in which they were laid as part of the experimentally extended clutches

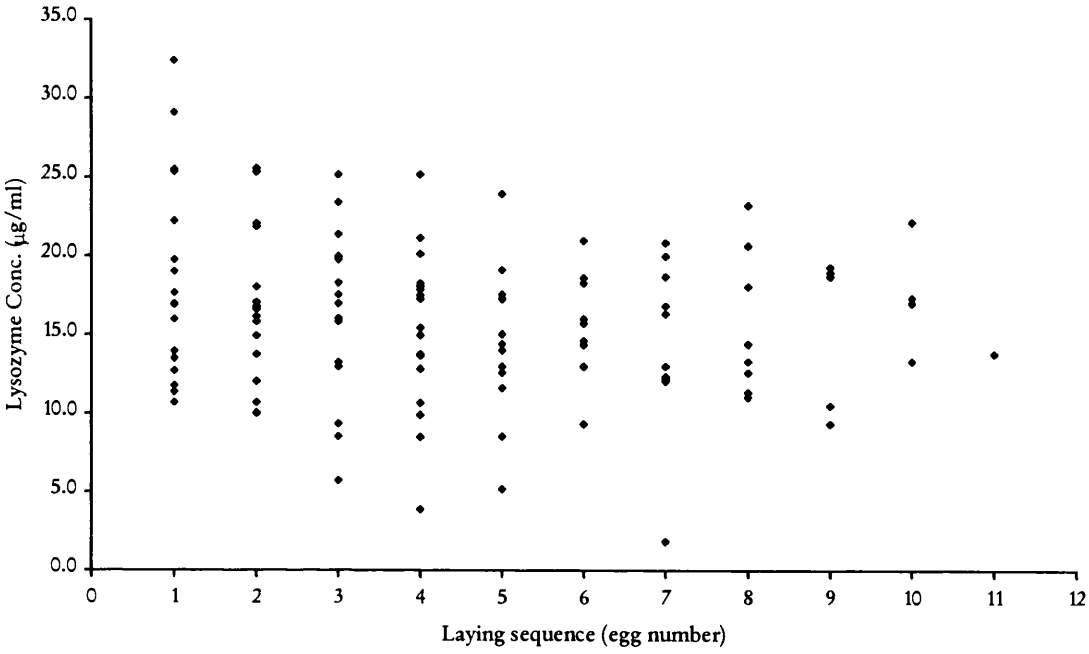
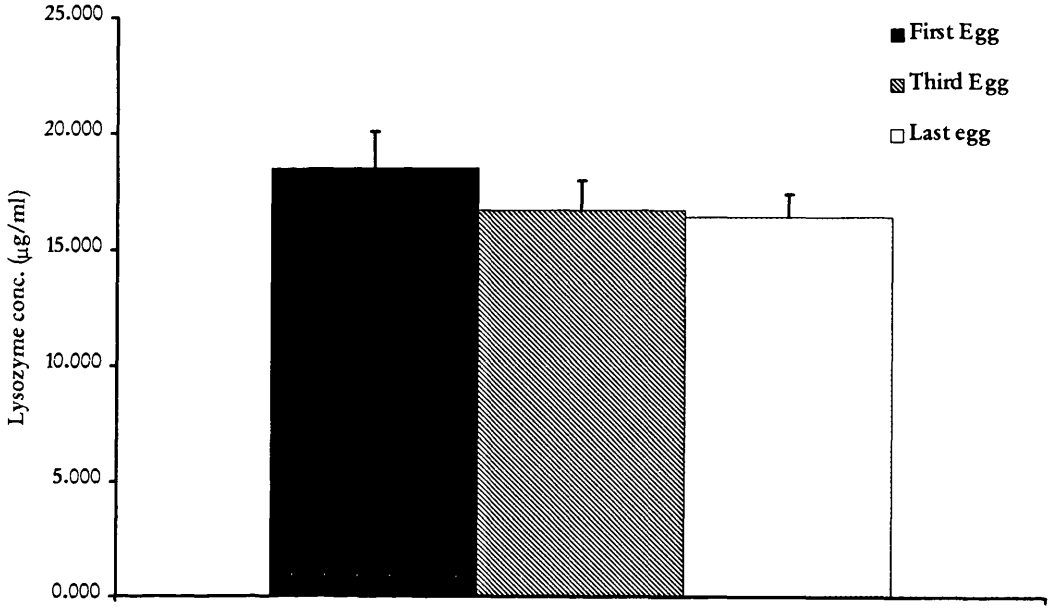


Figure 6.7 Mean lysozyme concentration (± s.e.) for first, third and last eggs in experimentally extended clutches (repeated measures ANOVA, $F_{2,16} = 1.24$, $p = 0.298$).



CHAPTER 7 Nutritional aspects of breeding in birds: general discussion

(Referring to a chicken egg) *"This egg type is, to us, a familiar and hence seemingly prosaic structure suitable for the breakfast table. It is, in reality, the most marvellous single 'invention' in the whole history of vertebrate life."*

Alfred S. Romer, 1968

My research addressed the complex relationship between dietary nutrition, female condition and reproduction as manifested via egg production and composition. Given the paucity of information available for species other than *Gallus gallus domesticus* I used quite diverse methods to achieve my objective. This involved experimental manipulations of egg production in captive and wild birds (cockatiels and lesser black-backed gulls respectively), and expansion of the composition data set by collecting eggs from a wide range of wild bird species for comparative chemical analysis.

Female quality, diet & reproductive resource allocation

For the purpose of this discussion, the mechanism by which laying birds meet the increased nutritional requirements of egg production can be reduced to whether females rely on capital investment (body reserves or stores), versus daily income (increased dietary intake, modified metabolism) to supply nutrients for the clutch. Feeding a supplement of high quality nutrients to cockatiels had a positive influence on egg production (Chapter 2). Clutch mass was 32% larger in birds that received the supplement all through the breeding cycle, compared to the birds who only received a maintenance diet over the same time period. Clutch size, rather than egg size, was increased in supplementary-fed females. High quality nutrition offered only during the period of egg formation did not produce significantly larger clutches. Instead there was a significant increase in clutch mass when a feeding supplement was offered only during the pre-laying period, suggesting an important contribution from endogenous reserves to egg production. Feeding a supplement of high quality nutrients did not appear to affect egg mass, an attribute often used as a measure of egg quality (Starck and Ricklefs, 1998). Chicks hatching from eggs laid by birds fed a supplement of high quality nutrients tended to gain weight and grow skeletally faster than those hatching from eggs laid by birds not fed the high-quality diet. This growth was not

related to the quality of the parents because of cross-fostering, therefore a real effect of egg composition was observed

Therefore the nutritional quality of the diet offered to cockatiels long before the laying period can have a profound influence on egg production and offspring quality. Research conducted on gulls and zebra finches suggests that the body condition of a female, as determined by her dietary experience in the weeks preceding breeding, influences her egg production (Bolton et al., 1992; Ramsay and Houston, 1997). Furthermore, egg formation has been shown to affect body condition at the end of laying (Selman and Houston, 1996; Nager et al., 2000), which in turn impacts on her future fitness (Nager et al., 2001; Visser and Lessells, 2001). The mechanism by which improved nutrient resources is allocated with respect to reproduction is as yet unclear (and beyond the scope of this investigation).

Which nutrients? Interspecific variation in egg composition

To understand which nutrients may be limiting egg production it would help to know what nutrients are actually present in eggs, and in what quantity. Eggs of 18 bird species were collected and analysed to examine interspecific variation in egg composition (Chapter 3). Where the data were available, I found the macronutrients (lipid, protein and water contents) of eggs closely matched values already published for these species(Ar and Yom-Tov, 1978; Carey et al., 1980; Sotherland and Rahn, 1987). Analysis of interspecific variation in egg amino acid profiles of 18 species represents the largest number and broadest coverage of avian orders sampled in any single study. After correcting for differences in egg mass, amino acid profiles in yolk appeared to be highly conserved across the range of bird species sampled. Certain albumen amino acids, methionine, phenylalanine and glutamic acid, were correlated with developmental maturity at hatching. The concentration of these amino acids was found to increase significantly in the eggs of species which produce more precocial offspring. Methionine, as a sulphur-rich amino acid functions as a precursor for cystine, which in turn is an important component of feather synthesis (Klasing, 1998)and precocity is partially characterised by degree of plumage development at hatching (Starck and Ricklefs, 1998).

Two fatty acids (linoleic and linolenic) are considered essential for all animals because they cannot be synthesised *de novo*, and a further three (arachidonic, eicosapenatoic and docosahexanoic) might be essential fatty acids, but their metabolism is only poorly

understood for avian species other than galliformes (Klasing, 1998). The range of these essential fatty acid concentrations in the yolk was dramatically different. Linoleic acid exhibited the largest range of concentrations between species, but in contrast to the amino acid results described above, this bore no relationship to the mode of developmental maturity. Linoleic acid is common in green plants and some seed oils, such as sunflower and safflower (Robbins, 1993) and the highest level of the this fatty acid was measured in eggs of predominantly herbivorous species. Thus, yolk fatty acid profiles differ between species as a result of their different diet, a conclusion also reached by Christy and Moore (1972).

Thus, addition to obvious diversity in gross egg composition between species, more subtle variation in composition exists which can potentially be explained by differences in developmental maturity at hatching and influence of maternal diet.

Intraspecific variation in egg composition – are 'specific' nutrients important?

Variation in egg composition, and therefore quality, also occurs within a species and this was examined in detail by manipulating the egg production of lesser black-backed gulls *Larus fuscus* (Chapters 4-6). Nager et al., (2000) demonstrated that in this species, egg quality differs along extended laying sequences. Birds were experimentally induced to lay extended clutches, representing the physiological and nutritional extremes of egg production.

On average, birds were able to produce three times the normal clutch size and egg mass declined significantly over the laying sequence. However weight of the last-laid egg did not fall below the weight of the last egg in a normal three-egg clutch. The weight of amino and fatty acids declined in absolute terms within an extended sequence (Chapter 5), but relative to egg mass remained at the same concentration. Earlier laid eggs contained significantly greater quantities of vitamin E and carotenoids, a phenomenon also observed in normal three-egg clutches (Royle et al., 1999). Once size has been corrected for, smaller eggs contained most macro nutrients (protein, lipid, water) in the same proportions as larger eggs, suggesting a blueprint for egg composition exists within the female, with limited scope for variation. That the last egg laid in extended clutches was not smaller than third eggs laid in normal clutches, indicates the probability of a minimum size threshold below which an egg is unlikely to hatch and survive. Carotenoids and vitamin E can be classed as

micronutrients since they are found in very small quantities within eggs and they are both powerful antioxidants that protect against peroxidative damage during development and the oxidative stress associated with hatching (Surai et al., 2001b). Caroteniods are also believed to contribute towards enhanced function of the immune system. Thus, differential mortality of chicks hatching from eggs laid later in a sequence may result from them having suffered more oxidative stress during development, or having an increased susceptibility to pathogens.

This hypothesis is bolstered by the finding that last-laid eggs contained significantly less immunoglobulin G (IgG) than earlier laid eggs (Chapter 5). The efficacy of immunological assays to measure variation in yolk immunoglobulins, the avian equivalent of maternally-derived passive immunity was determined first (Chapter 4). An enzyme-linked immunosorbent assay (ELISA) proved the most appropriate, due to the level of accuracy, repeatability of results and its' sensitivity to detect variation in immunoglobulin titre in egg yolks along extended laying sequences. Furthermore later-laid eggs of experimentally extended clutches laid by *L. fuscus* showed lower lysozyme activity than earlier-laid eggs (Chapter 6). Since egg mass also declined significantly with laying position, these later-laid eggs were also more likely to contain less lysozyme in absolute terms. Lysozyme is present in the egg albumen and along with immunoglobulins is another 'non-nutritive' element of egg composition, believed to confer protection against bacterial contamination. On its own, the decreasing lysozyme concentration observed in experimentally extended gull egg clutches may not be biologically significant because we do not know how lysozyme activity relates to offspring survival in the wild. There do not appear to be many bacteria pathogenic to vertebrates which are susceptible to lysis by lysozyme, and Chipman and Sharon (1969) speculate that lysozyme serves to digest cell wall debris from bacteria killed in other ways. This idea is supported by research suggesting a close functional relationship between lysozyme and specific immunoglobulins connected with bacteriolysis (Jollès and Jollès, 1984). Therefore, combined with declines in other components of the egg already described, reductions in lysozyme concentration may contribute cumulatively to the decreased hatchability & fledging success observed in previous research (Nager et al., 2000).

Outlook

Our knowledge of poultry nutrition is relatively mature, including very complete listings of required nutrients, quantifying minimal levels needed to maximise production characteristics (e.g. egg laying) and bioavailabilities of essential nutrients supplied by commercial feedstuffs (NRC, 1994; Klasing and Leshchinsky, 1999). Indeed it has provided the benchmarks to which most of the data collected within this thesis have been compared. Yet it is not known or understood whether the requirement values that are recognised to maximise commercial productivity (growth and egg laying) in relatively healthy, unchallenged animals, are optimal for growth, development and reproduction in non-domesticated avian species either being maintained in captivity, or found in the wild.

There is evidence that some avian species are capable of making dietary shifts during the reproductive cycle, for example pipit (*Anthus*) species selecting calcium-rich items to compensate for deficiency in acidified areas (Bureš et al., 2001). However in many cases it is not clear whether this is to satisfy specific nutrient requirements or reflects changes in food availability (Perrins, 1991). Certainly captive birds can only choose from what they are provided and are dependant on that diet being nutritionally adequate, based on the species' likely requirements. As an order, the Psittaciformes contain a significant number of threatened species (Bennett and Owens, 1997), many of which are the focus of captive breeding programmes (Wilkinson et al., 2000). This single avian taxa is further distinguished by comprising of species with an extremely diverse nutritional ecology: from almost exclusively nectarivorous species e.g. swift parrot (*Lathamus discolor*) and musk lorikeet (*Glossopsitta concinna*), to highly frugivorous species e.g. Pesquet's parrot (*Psittrichas fulgidus*) and also highly herbivorous species, e.g. kakapo *Strigops habroptilus* (Powlesland et al., 1992; Gatrell and Jones, 2001; Pryor et al., 2001). Even the traditional image of parrots, as seed-eaters or granivores, in fact ranges from the smallest psittacines, budgerigars and cockatiels to the largest macaws *Ara* sp. (Juniper and Parr, 1998), consuming food items with very different nutrient composition.

Much of our knowledge of food composition centres around those items directly consumed by humans or commonly used as feed for domesticated animals. While detailed natural history documentation of field biologists supplies a written record of items consumed by many wild psittacine species (e.g. Wermundsen, 1997; Renton, 2001), such information, with no chemical evaluation of dietary consituents, or assessment of

utilisation, provides only a partial basis for applied feeding programmes. This is a critical consideration given the reliance on captive birds to act as a reproductive reservoir for threatened species (Wilkinson et al., 2000). Returning to some of the specialist psittacine consumers already mentioned, eucalyptus pollen appears to be an important source of protein for the nectarivorous species, swift parrot and musk lorikeet (Gatrell and Jones, 2001). Pesquet's parrot appears to have reduced protein requirements in order to accommodate a highly frugivorous diet, a strategy shared with another almost exclusive fruit-eating bird, the cedar waxwing (*Bombycilla cedrorum*) (Pryor et al., 2001). Further considerations relevant for captivity are that the capacity of parrots to digest seed fibre changes according to the age of the bird (Vendramin-Gallo et al., 2001).

Micronutrients may have macro-consequences within birds' diets, yet little is known about micronutrient requirements or the evolution of plant interaction with a micronutrient-poor ecology (Levey and Martinez del Rio, 2001). Plants may take advantage and attract a diverse array of seed dispersers by offering an essential, but scarce micronutrient e.g. high calcium content of some tropical fig species (O'Brien et al, 1998).

Avian eggs "contain all the chemical nutrients needed for the growth of the embryo." Burley and Vadehra (1989, p1). This all needs to be in place before the embryo starts to develop and be sufficient to cover the entire period of embryogenesis until hatching. The deposition of appropriate nutrients *in ovo* by a female bird represents a considerable commitment of her resources to reproduction (as reviewed by Monaghan and Nager, 1997). Variation, in both the total amount of resources allocated to a clutch of eggs and the distribution of those resources within a clutch, can clearly have a profound influence on both the offspring and the female's own fitness. Although we know much about the chemistry and physiology of the avian egg (Romanoff and Romanoff, 1949; Burley and Vadehra 1989), it is primarily via eggs of domesticated poultry, dominated by one species in particular *Gallus gallus domesticus*. While this has served as an excellent model there are obvious limitations. The chicken is a granivorous bird, producing precocial young and its eggs are either considered as food for human consumption, or as a mechanism for sustaining intensive production systems – neither of which are particularly relevant for understanding the reproductive strategies of wild birds.

Experimentally extended clutches may not seem a common natural occurrence, but the practice of manipulation egg production is used in captive breeding programmes for endangered bird species (Jones and Swinnerton, 1997). The data presented in this thesis highlight the problem of manipulating egg clutches when we don't really know what effect we are having. Captive diets have been found to alter the egg yolk lipids of American kestrel *Falco sparverius* and red-legged partridge *Alectoris rufa* and, in extreme cases, the authors speculate this could possibly have deleterious consequences for the development of the embryo and neonate (Surai et al., 2001c). The composition of yolk lipids may prove particularly vital for other species, such as the King penguin *Aptendoytes patagonicus*, whose natural diet is high in polyunsaturated fats that are highly susceptible to peroxidative damage. Appropriate fat-soluble vitamins (of dietary origin) are transfered from yolk to embryo to compensate (Surai et al., 2001d). What controls egg production and the trade-offs in resource allocation females are making when pushed beyond normal limits is still unclear. But not all eggs are equal, and although general food provision may be adequate for extra egg production, without due care and attention to the type of supplementation eggs may be impoverished in some small but critical respect.

Returning to within-clutch variation in egg composition, does nutrient allocation differ between male and female offspring? We know that for the lesser black-backed gull, sons hatching from poor quality eggs are less likely to fledge than daughters hatched from poor quality eggs (Nager et al., 1999); the reverse is true in the great skua *Catharacta ana*, aspecies with reversed sexual size dimorphism (Kalmbach et al., 2002). In some species the male and female eggs differ in size e.g. American kestrels *Falco sparverius* (Anderson et al., 1997); house sparrow *Passer domesticus*, (Cordero et al., 2000) and mountain white-crowned sparrows *Zonotrichia leucophrys oriantha* (Mead et al., 1987). Do sons and daughters differ in nutrient requirements? If so, is this simply a size effect or due to more subtle variations in chemical composition? Controlled either directly by hormone concentrations present in the egg or mediated by nutrient-dependant gene expression of these hormones? In many species androgen in yolk varies with laying order which in turn may affect hatching muscle development (Lipar, 2001). Differential nutritional investment in eggs may also confer disease resistance against nest parasites (Saino et al., 2001). Do these phenomena represent a deliberate positive boost to the developing embryo, or is it simply a passive consequence of the egg formation process?

Required nutrients are commonly regarded simply as substrates for the synthesis of macromolecules, or for the provision of energy. Yet these substrate functions of many nutrients may not be the only, or even the most important, mechanism for which the nutrient is 'required'. Gene expression of some insulin-like growth factor (IGF) binding proteins in Japanese quail *Coturnix japonica* appear to be under the control of vitamin A status; in turn, the IGF system appears to mediate the physiological actions of vitamin A in determining the quail's growth (Fu et al., 2001). Nutrient concentrations can also affect tissue development. Insufficient vitamin A influences functionality of the chicken small intestine by altering proliferation and maturation of cells in the intestinal mucosa, thereby interfering with normal growth by reducing the digestive efficiency of the bird (Uni et al., 2001). Furthermore, individual nutrients rarely act in isolation so despite descriptions throughout my thesis of individual levels of specific nutrients, they most certainly should be considered in relation to other compounds. For example, supplementation with either vitamin E or the mineral selenium in the maternal diet has been demonstrated to have a significant positive effect on the antioxidant system of her offspring, but this effect was further increased using a combination of the compounds (Surai, 2000).

These investigations of egg production and the chemical composition of eggs, both within and between species, demonstrate the complexity of the avian female's reproductive system. Female body condition is an integral part of that system and it would appear to influence egg production long before oviposition. While knowledge of poultry nutrition is relatively mature, clearly our understanding of comparative avian nutrition and the strategies evolved by wild bird species is still in its infancy.

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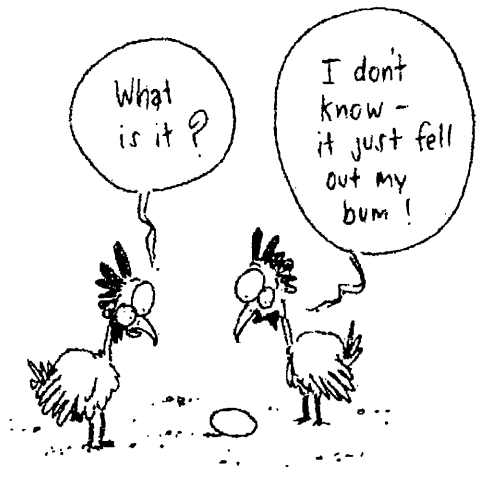

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APPENDICES

Appendix I Determination of Moisture and Ash by the LECO thermogravimetric analysis (TGA501)

Appendix II Determination of nitrogen and protein using the LECO FP428 analyser

Appendix III Determination of total amino acid profile

Appendix IV Extraction of total fat by Bligh & Dyer

Appendix I Determination of Moisture and Ash by the LECO thermogravimetric analysis (TGA501)

Moisture and Ash

The TGA501 is a multiple sample determinator and is used for determining the composition of organic, inorganic and synthetic materials. It measures weight loss as a function of temperature in a controlled environment. The unit consists of an electronics unit for furnace control and data management and a multiple sample furnace, which allows up to 19 samples to be analysed simultaneously.

After an analysis profile has been selected, empty crucibles are loaded into the furnace turntable. The profile controls the turntable operation, locating crucible position and establishing crucible tare. On completion of crucible tare, each crucible is presented to the operator for sample loading. The starting weight is measured and stored automatically. Once all the crucibles have been loaded, analysis begins. The weight loss of each sample is monitored and the furnace temperature is controlled according to the slected analysis parameters. The percent weight loss in each sample for each analysis step is printed at the end of the analysis.

The analyser contains an easy to follow, menu-driven programme that allows analysis parameters to be tailored to satisfy most analytical applications. Located in the furnace is a 19 position sample turntable together with a balance pan. Samples of 1-3 grams may be weighed in the crucible. Also in the furnace is an empty crucible which is weighed and is designed to compensate for balance drift.

All weighing is performed automatically. The furnace uses an atmosphere of air from the compressor but can be set up to use either oxygen or nitrogen. Three electrical heating elements ensure uniform sample heating.

Moisture was determined as the loss on drying at 105 °C for 5 hours. Total Ash was measured after heating samples to 550 °C, following the drying procedure described above.

Appendix II Determination of nitrogen and protein using the LECO FP428 analyser

Crude protein

This method provides an accurate means for the determination of protein content using the Dumas principle instead of Kjeldahl, which will only determine nitrogen from organic sources. This procedure will detect and measure nitrogen in all forms.

The FP428 is a microprocessor based, software-controlled instrument that determines the nitrogen in a variety of materials. There are three main phases during an analysis cycle. These are purge, burn and analyse. In the sample Drop Purge Phase, the encapsulated samples is placed in the head and sealed. At the same time the loading head is purged of any atmospheric gases that have entered during sample loading.

In the Burn Phase, the sample is dropped into a hot furnace (850 °C) and flushed with pure oxygen for very rapid combustion. The products of combustion, mainly carbon dioxide, water, nitrous oxide and nitrogen are passed through thermoelectric cooler to remove most of the water. The remaining products are collected in the ballast volume. All the gas products in the ballast volume are allowed to become a homogenous mixture at a pressure of 975 mm and a constant temperature.

In the Analyse Phase, the piston in the ballast volume us forced down and a 10cc aliquot of the sample mixture is collected. The sample aliquot is passed through hot copper to remove oxygen and to convert nitrous oxide to nitrogen. The aliquot is then passed through Lecosorb and Anhydrone to remove carbon dioxide and water respectively. The remaining combustion product is nitrogen and this is measured by the thermal conductivity cell. The final result can be displayed as percent nitrogen or protein, using the nitrogen conversion factor of 6.25 (i.e. nitrogen value x 6.25).

Appendix III Determination of total amino acid profile

Total amino acid profile

This assay would generally be used to assess protein quality.

The sample is hydrolysed by refluxing with 6M Hydrochloric acid for 23 hours. This procedure breaks down the protein chain into its individual amino acids. However during the hydrolysis amino acids are degraded to some degree. Those to be specifically considered are the sulphur-containing amino acids, cystine and methionine[1], which are partially degraded and tryptophan which is totally degraded. They must be analysed separately by different methods – cystine by performic acid oxidation and tryptophan by alkaline hydrolysis.

Nor-leucine is added as an internal standard at the start of the hydrolysis. The pH is adjusted, the solution diluted with loading buffer loaded onto the LKB amino-acid analyser. The individual amino-acids are separated by ion-exchange chromatography and quantified by comparison with an internal standard. This process enables the quantitative determination of the following amino acids:
Aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, valine, methionine, iso-leucine, leucine, tyrosine, phenylalanine, histidine, lysine and arginine.

When reporting amino acid data CNL includes a recovery factor based on the summation of all individual amino acids as percentage of the crude nitrogen x 6.25. An acceptable range for this recovery is 85-110%.

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[1] Note that although methionine is reputed to be degraded by hydrolysis, the experience at CNL over many years is that this is not the case and more importantly they find higher answers by total amino acid profiling than by performic oxidation and recommend that this figure is the most accurate in terms of nutritional status.

Appendix IV Extraction of total fat by Bligh & Dyer

Total fat

This method extracts free and bound lipids from materials without altering the chemical structure of the fat and therefore it is useful in extracting fat for examination by further analytical techniques to give information concerning its composition or condition. The fat is extracted using a mixture of chloroform, methanol and water. An aliquot of the chloroform layer is evaporated to dryness and the remaining fat weighed.

Fatty acid profile by Gas Chromatography

After lipid has been extracted by the Bligh & Dyer extraction method described above Nonadecanoic acid (C19:0), which doesn't usually appear in food profiles, is added as an internal standard. This mixture is saponified with methanolic sodium hydroxide to hydrolyse the glycerol/fatty acid ester linkage. The fatty acids are then converted to their methyl esters using Boron trifluoride/methanol complex, which are subsequently partitioned in Heptane for assay by gas chromotography. The methylation of the fatty acids renders them more suitable to analysis by gas chromatography. This technique is so universal for fatty acids that it is commonly abbreviated to the acronym FAME analysis – Fatty Acid Methyl Ester(s).